



Volume 249

June 28, 1974

## and now . . . nuclear hypocrisy in Britain

It has been a quite remarkable two months in which the issue of nuclear weapons testing has emerged from the shadows in which it has been lurking for ten years. The United States and Soviet Union have made noises about a further treaty; India has detonated her first device; China has fired another one (her sixteenth); France has ventured on a new series. It was only Britain of the nuclear powers who had not contributed to the drama, and now she too has obliged in characteristic fashion—by being found out.

The revelation by Mr Chapman Pincher in the *Daily Express* that there was shortly to be a British test in Nevada was greeted officially by refusals either to confirm or deny. Now Mr Wilson has announced that the test has already taken place. He must have strong feelings of *déjà vu*, as he found himself with a similar legacy from the Conservative government in 1965 when he last stopped Britain's testing activities.

Since this test surfaced as the result of a leak—and not a government orchestrated one at that—one is bound to ask whether this really is the first for nine years or whether previous testing in Nevada has been kept more effectively behind a security screen. There is really no way that anyone can find out, as testing in Nevada goes on so regularly that there would be nothing untoward to the outside world about the occasional additional test. Obviously whilst Mr Wilson was in power in the sixties there would be ideological objections, but Mr Heath was Prime Minister for nearly four years and would have no commitment to self-restraint. It seems inconceivable that it would take four years from the brakes being released until as vigorous an organisation as Aldermaston could find time to test.

The tragedy of the recent revelations is that this British obsession for secrecy in all matters of defence has rebounded so vigorously on prospects for British diplomacy. A few weeks ago *Nature*, along with almost everyone else in the West, was rebuking India for becoming a nuclear power, and the main line that most, including ourselves, pursued was that it was simply hypocritical to talk of a nuclear explosive as capable of being labelled peaceful. Britain, of course, could be particularly smug since she had renounced the provocative gesture of firing nuclear devices years ago. Our restraint was an example to the world. Now we find ourselves with much the same sort of hypocrisy on our doorsteps—a failure to be at least elementally honest about nuclear affairs; worse, a willingness to have our own test labelled, by default, as American. It is difficult

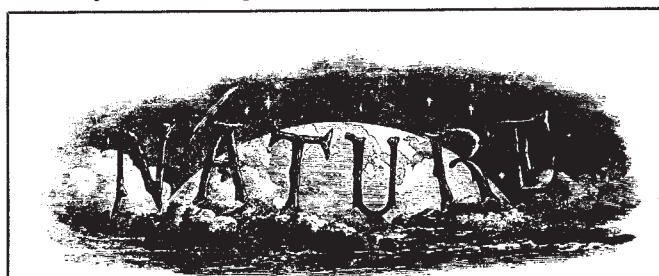
to see why secrecy should extend this far. Obviously the Labour Government were not going to reveal the hot potato they were passed, but surely the Conservative government would have been in no danger in giving prior notice. The announcement of a test would barely have stirred a ripple; the discovery is bound to make waves.

The real significance of recent events, however, is that they show how inextricably intertwined are British and American nuclear interests. It is just not possible for one country to detonate explosions at another country's facility without there being such a high degree of liaison that one is bound to conclude that the two countries act as one in nuclear matters. Of course, the possession of Polaris submarines already ensures this to a large extent; the nuclear test simply brings it once more to our attention. This liaison reduces Britain's independence in the discussion of nuclear issues on a world stage to an ineffectual level. This is very unfortunate at a time when the two superpowers appear to be on the verge of agreeing between themselves on some form of nuclear test treaty.

The form most widely discussed is of a threshold agreement, restricting tests not to exceed a certain seismic magnitude. There have been strident objections to this throughout the scientific community, based on a feeling that such a treaty is easy to find loopholes in, leaves room for ambiguity and has not been given a fair debate in wide enough a community. It is possible to sense qualms in British circles too, but what hope is there of an independent and questioning line under these circumstances?

The entanglement, however, does not simply constrain Britain in nuclear matters. It is impossible to see how Atlantic and European relationships on all diplomatic matters can fail to be influenced to a degree by the nuclear commitment. It is high time that the nuclear relationship and its influence on foreign policy was looked at with great care.

## 100 years ago



MR. JAMES LICK, of San Francisco, California, having in the course of his life accumulated a large fortune, has recently concluded a deed by which he conveys all his property to seven persons upon trust to be applied to various worthy objects. Among these, 700,000 dols. are to be applied to the construction of a more powerful telescope than any yet made, to be erected at an observatory in California, and 300,000 dols. to found, in California, a school of the mechanical arts.

From *Nature*, 10, 171, July 2, 1874.



## Kill a Pinta goat a day . . .

*Science and conservation in the Galapagos Archipelago must swim against a fast running tide of tourism and goats. David Davies looks at the way in which the Ecuadorian Government and the Charles Darwin Foundation are trying to maintain the islands.*

"I FOUND long ago that I cannot live happily without the Galapagos Islands; even if I am not there I need to know that they exist undestroyed by goats or men . . ." So wrote Peter Kramer, retiring director of the Charles Darwin Station on Santa Cruz in the Galapagos Islands in December 1973.

The Galapagos Archipelago is on the equator about 1,400 kilometres off the Ecuadorian coast. Darwin was there for 35 days in 1835, and was deeply influenced by what he saw. His field notes were dominantly geological; he was struck by the youthful volcanic nature of the rocks. It was however, the biological scene which was ultimately to have most impact on him—giant tortoises, finches (Darwin's finches) whose bills were "modified for different ends", marine iguanas, flightless cormorants and many others. By the time Darwin was writing *Origin of Species* he had absorbed much else and the effect of his stay in the Galapagos is less obvious, but in his early evolutionary writings the significance of islands is crystal clear; in 1842 he wrote on the back of a notepad "islands are the nursery of new species".

He was not the only visitor to the Galapagos. In the nineteenth century whalers would put in on their long journey from the Pacific to the American East Coast round Cape Horn, and would collect a giant tortoise to keep in the hold of the ship for later consumption. Many other ships also put in to the Galapagos, caring nothing about what species they brought in. One such import about seventy years ago was the Norwegian black rat which had such a grip on Pinzon, for example, that by 1965 no tortoises younger than thirty could be found; all tortoise hatchlings were being killed by rats. Other feral animals such as the pig have also wrought havoc on the tortoise population of various islands by eating the eggs. But the most dramatic and visible introduction has been the goat.

The local population has been in the habit of depositing goats on the more remote islands to ensure a food supply for fishing expeditions. Thus in 1958, a male and two females were left on Pinta. By 1968 the number had reached 4,000 to 5,000 and in 1971 the estimate was "not below 20,000". Hardly surprisingly, species of plants unique to the archipelago are threatened by this population on Pinta and elsewhere.

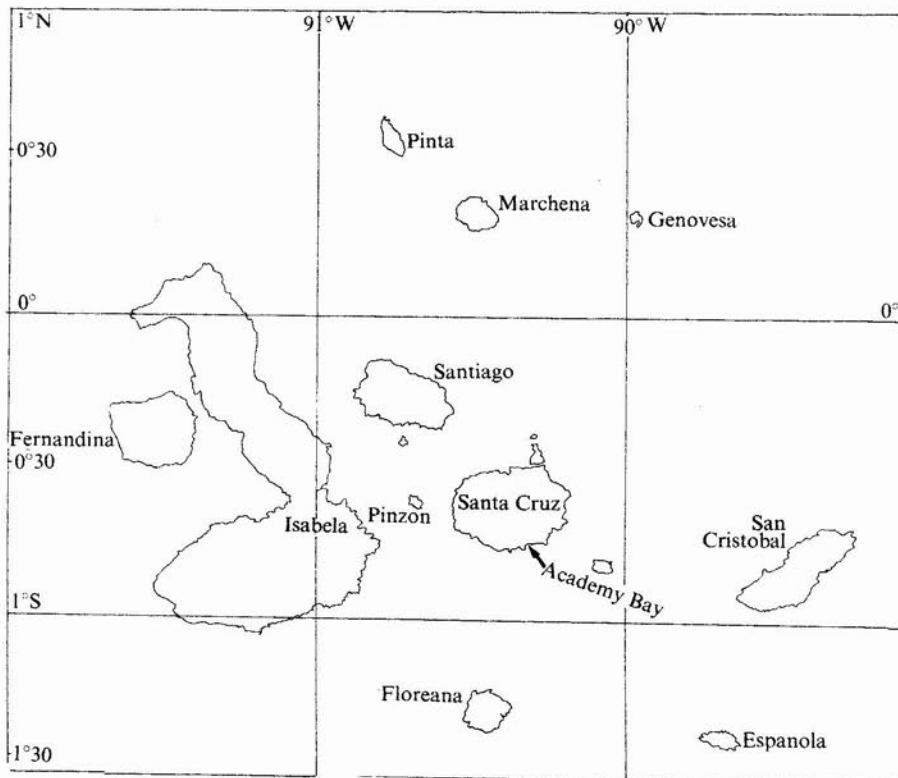
Not all the impact on the Galapagan environment has come from man's works. There are many historically active volcanoes in the west of the archipelago, and in 1968 the caldera collapse of Fernandina was the world's largest collapse in seventy years. In addition latitudinal oscillations of the Oceanic Equatorial Counter Current (the 'El Niño' phenomenon) lead to long term fluctuations in rainfall and have striking effects on the life of the islands. The most recent threat, however, has been man himself, as resident and tourist.

The population of the islands was less than 1,000 in 1960; now it is more than 4,000. Santa Cruz and San Cristobal have the most inhabitants whilst some islands, particularly the active volcanoes, are prudently uninhabited. Many of the inhabitants are farmers but an increasing number are needed

to service a tourist industry which now provides for several thousand annually compared with 200 in 1969. Much of this tourism has been stimulated by the natural environment of the Galapagos and has been appropriately careful, but a growing number of private yachts visit the island on Pacific cruises and they are causing serious problems. As one scientist put it, "man has now replaced goats as the greatest threat".

The conservation issue is not simple. Although biologists would agree that there is an obvious case for the strictest of measures to preserve the islands intact, they are also the home of many Ecuadorians. In addition they occupy a strategic naval position. In two World Wars it was "he who holds the Galapagos holds the Panama Canal". There is thus a strong naval presence and in the past has even been some suspicion by the military that scientific operations have had more sinister overtones. There is thus a delicate diplomatic balance necessary in order that residents, military, tourists and nature coexist.

Ecuador has had a formal interest in Galapagan conservation since 1934 when legislation was enacted to ban the export of protected animals and the import of domestic ones. Nature reserves were also established, but

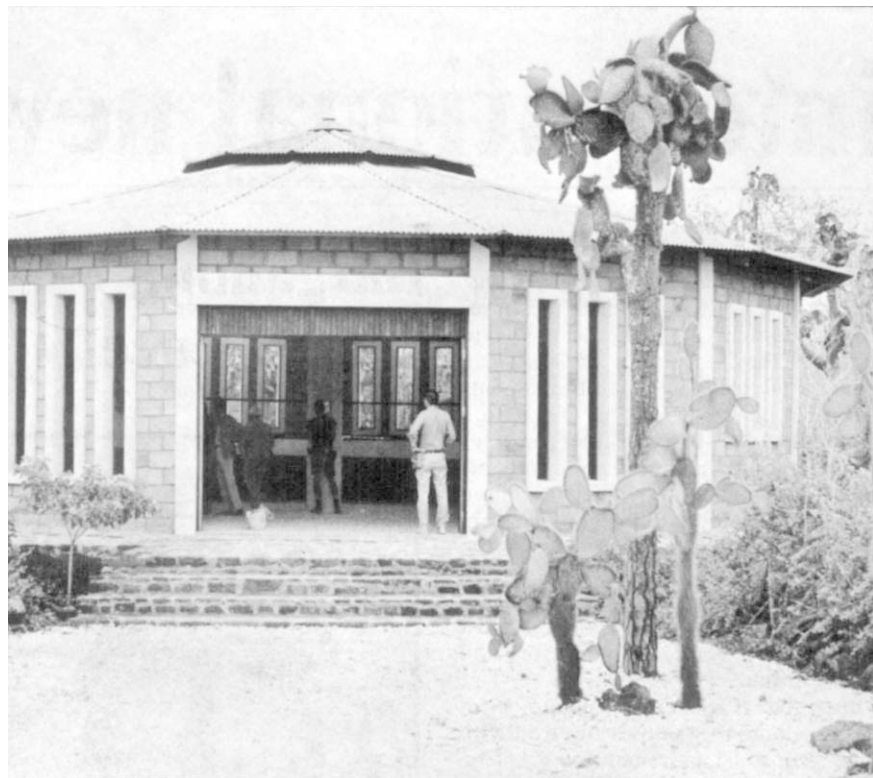




*Tortoise house on Santa Cruz:  
keeping the rats at bay*

the legislation was ineffective as there was no organisation to enforce them. An American effort to set up a scientific station in 1941 was shelved because of the war, and it was not until 1959 that a Charles Darwin Foundation for the Galapagos Isles was formed in Belgium with the aim of constructing a research station on Santa Cruz. Also in 1959 Ecuador declared the whole archipelago (save four small inhabited areas) a national park. The Galapagos National Park Service now has nine officials and wardens and will grow with the revenue from a levy on tourists. A much talked-about event in 1971 was the removal and resettlement of fifteen families living illegally in park territory. This was seen as unequivocal evidence that the government would even act against its own subjects in maintenance of the park. In addition poaching has been cut down and undesirable tourist schemes stopped. The goats and pigs are under control in the smaller islands although there is an enormous amount of work still to be done on the larger islands. Most important, there is a close collaborative arrangement with the Darwin Foundation. A concern voiced by the Forestry Service, however, is that it will not be possible much longer for Galapaguenos to live with a scientific research station and a tourist industry which constrict their freedom without sharing the income.

The Charles Darwin Research Station at Academy Bay has been the staging post for research since the early 1960s. UNESCO has paid for the director (the present one is Craig Macfarland, whose primary scientific interest is the tortoise). Building of a laboratory, a house for the director and accommodation for visitors was finished by 1963. Once the extent of the depredations of the rats on Pinzon's tortoises had been discovered and some success achieved with rearing at the station, the San Diego Zoological Society helped provide a special building (see picture) for tortoise rearing. A few other buildings are now also in the complex. The staff comprises, in addition to the director, one or two additional scientists and maintenance and service workers. A yacht, *Beagle III* is also operated. It would be foolish to pretend, however, that the station is a great social and scientific centre. It is generally regarded by visitors as adequate in basic facilities for support-



ing up to twelve visitors, but several scientists speak of the depressing environment. "Like an unmanned youth hostel" was one remark. "The station has to spend much of its income shooting goats and standing guard" was another. It gets just one journal—a complimentary *Nature*.

Up till now the Charles Darwin Foundation has operated entirely on a voluntary basis. It has officers both in Europe and America all of whom give their services free; several institutions either pay for a research table or donate funds but UNESCO support for the director will end this summer. The World Wildlife Fund has been funneling some funds to the station. The Smithsonian in Washington provides secretarial assistance and the services of one of its staff, Tom Simkin, as Secretary for the Americas. And many individual friends of the Galapagos donate. At least \$100,000 is needed annually just to keep the station alive; much more for it to pursue the more stimulating and imaginative programmes it would wish to do, in particular the education of young Ecuadorians. It is going to be increasingly difficult for a research station to exist without providing tangible educational benefits to Ecuador. At present there are programmes for Galapagan residents and teachers, wardens and students from the mainland, but there is a widespread feeling that much more needs to be done to draw the Ecuadorian scientific community into the station's activities, and this means being able to provide for university

students and give them a taste of biological research—something that they will otherwise find difficult to get without emigrating.

The question that now has to be asked is whether the Charles Darwin Foundation can continue its funding, and hence its programmes, in a piecemeal fashion dependent on the goodwill and hard work of unpaid officers. Peter Kramer was particularly adept at fund raising and last year organised a benefit cruise to the Galapagos which brought in \$50,000. But this year's attempted repeat failed to get the patronage, the cruise was cancelled and the deposit lost. "It's partly the change in economic climate," says Simkin, "but to be honest, we just didn't hustle enough; we are full-time scientists inexperienced in the art of persuading". The attractions of an organisation with no overheads at all have soon to be weighed against the need for a continual expansion of scientific, conservation and education programmes which necessarily requires some professionalism on the financial side, particularly since the director's salary now has to be paid by the foundation. There is a divergence between Europe and the United States in approach. The Europeans on the foundation are more prepared to take financial setbacks and consequent delays phlegmatically whereas the Americans seem to be increasingly concerned that delay and a continued environment of poverty for the director and visiting scientists could lead to permanent loss.



# international news

PRESIDENT Nixon returned from his triumphal tour of the Middle East last week armed with a clutch of promises and agreements which once again underline the importance of American science and technology as instruments of foreign policy. During his five-nation spectacular, he dangled the prize of American-built nuclear power plants before both Israel and Egypt, promised an extensive programme of bilateral scientific exchanges between the United States and Egypt, Israel and Jordan, and reaffirmed a scientific co-operation agreement negotiated last month between the United States and Saudi Arabia.

But the President's technological diplomacy didn't go down too well with some people back home. Even before Nixon and his entourage had left the Middle East. A barrage of complaints was raised in Washington at the prospect of nuclear power being exported to such a volatile area, particularly in view of India's recent use of Canadian-supplied nuclear technology to manufacture an explosive device. Senator Henry M. Jackson called the agreement "dangerous". Senator Mike Gravel, an avowed nuclear critic, said that it would be "like exporting the black plague (sic)", and a raft of senators and congressmen have supported legislation aimed at giving Congress the final say on any international agreement involving the export of nuclear technology from the United States.

The nuclear agreements at this stage represent no more than an official commitment on the part of the United States to open negotiations with Israel and Egypt on the supply of reactors. The negotiations will not begin for several weeks and it will be months before final agreements are struck.

According to sources in the Atomic Energy Commission (AEC) and the State Department, the tentative proposal is for the United States to supply both Egypt and Israel with single 600 MW light water reactors which, if all goes according to plan, could begin operating in 1980 or soon thereafter.

For both Israel and Egypt, such an agreement would provide nuclear power for the first time, although both countries now operate small-scale research reactors. Israel has one 5 MW reactor, supplied by the United States in 1960, in operation at Nahal Soreq and another 26 MW reactor, supplied

## Bargaining with science

by Colin Norman, Washington



Nixon: Beirut magazine cover boy

by France in 1963, located at Dimona in the Negev Desert. Egypt has a single 2 MW reactor which was built by the Soviet Union in 1961.

News on the impending deal—which caught many congressional leaders completely by surprise—has raised fears, however, that the reactors could enable either country to acquire nuclear weapons. Such fears stem from the fact that light water reactors, during normal operation, produce plutonium, which can relatively easily be turned into the core of an atom bomb similar to the device which devastated Nagasaki in 1945.

According to two experts on the matter, Theodore Taylor and Mason Willrich, who recently wrote a book on nuclear safeguards, a light water reactor steadily delivering 1,000 MW of electricity produces about 250 kg of plutonium each year—about 10 kg is considered sufficient for one bomb. And the whole issue has been brought into sharp focus by the explosion in India last month of a device manufactured from plutonium supposedly obtained from a 40 MW research reactor supplied by Canada.

There is, however, good reason to suggest that the risk of introducing nuclear weapons into the Middle East by supplying power reactors to Israel and Egypt can be reduced to negligible proportions by a system of internation-

ally monitored safeguards. The key to the matter is what happens after the 'spent' fuel is removed from the reactors.

The United States is bound by international law—the Nuclear Non-proliferation Treaty (NPT)—to put reactors which it supplies to other countries under the control of the International Atomic Energy Agency (IAEA) which conducts inspections and monitors the operation of the reactors to make sure that no fissile material is diverted into weapons programmes. In addition, it is likely that the United States will insist on extra safeguards in the case of the impending Middle East deals.

Essentially, the safeguards will be pretty simple to carry out. Nuclear fuel will be supplied from the United States in the form of slightly enriched uranium (uranium-238 containing about 3 per cent of the uranium-235 isotope), which could not be made into an atomic weapon. Thus there will be little chance of diversion into a weapons programme from the fuel supply end of the operation. Once the fuel has been sufficiently depleted in the reactor, it will be removed and probably stored for a few months in a concrete-lined pool at the reactor site to allow some of the radioactivity to decay, and then shipped to another country for reprocessing. The safeguard system will thus consist essentially of monitoring the fuel going into the reactor and the spent fuel rods coming out of it.

For either country to beat the safeguards system and manufacture an explosive, it would have to build a complex and expensive re-processing plant to recover plutonium from the spent fuel rods, and evade the monitoring system. That would be extraordinarily difficult to accomplish undetected. India managed it because it had a reactor which was not subject to international inspection, and it also has its own reprocessing plant. Thus plutonium was readily available for fabricating an explosive device, and the only safeguard was a commitment from Indian officials to use the stuff for 'peaceful purposes'.

One irony in the present flap about the possible Middle East nuclear deals is the fact that Israel is probably capable of manufacturing nuclear explosives in any case. The Dimona reactor was built by France with no known

safeguards or international inspection rights (it is in fact the only reactor apart from the Canadian-Indian plant which is unsafeguarded). According to representative Melvin Price, Chairman of the Congressional Joint Committee on Atomic Energy (JCAE), it is capable of producing 8 kg of plutonium a year, which is almost enough for a crude bomb. The country is not known to have constructed a plant for separating out the plutonium from the spent fuel rods, but according to one US official, it could do so relatively easily. Thus, whatever safeguards are applied to the proposed power reactor, Israel would still be capable of joining the nuclear club if she chooses to do so. It should also be noted that neither Israel nor Egypt has ratified the Non-proliferation Treaty, although Egypt has signed it.

Be that as it may, the nuclear deals are likely to be developed as follows. Israeli and Egyptian officials have been in Washington this week to negotiate contracts for the supply of fuel to the reactors. These negotiations, ironically, have to be completed before agreement is reached on the reactor sales because the Atomic Energy Commission has set a June 30 deadline for new contracts involving the delivery of enriched uranium to foreign countries in the early 1980s. Then, a few weeks later, negotiations will begin on the sale of the reactors themselves. If agreement is eventually reached

the JCAE will be informed and Congress will have 30 days in which to react. The agreement could be prevented from going into effect only if Congress passes a bill disapproving the terms under which the sale will take place. But the Bill would be subject to a possible presidential veto.

Representative Price has promised that the JCAE will give the matter a full and open review, and he has said that he "will keep (Congress) appropriately informed" of developments, during the negotiations.

But several members of Congress are dissatisfied with that arrangement, and are attempting to give Congress veto power over any international agreement involving the export of nuclear technology. Representative Lester Wolff has already introduced a Bill into the House which will prevent the Middle East agreements from going into effect until Congress gives its express approval of the deal, and Senator William Proxmire has announced his intention to propose an amendment to the Export Administration Act when it comes before the Senate in the next few weeks, which will require that "no nuclear technology, nuclear materials, or associated equipment could be sent to any country until both houses of Congress give approval in a vote". According to a staff member of the JCAE, however, such a requirement would place a huge burden on Congress because in the

past six months alone the United States has entered into some 50 separate agreements involving such transactions.

In contrast to the nuclear agreements, the scientific exchange proposals announced by President Nixon have scarcely raised a flicker of interest in the United States. As far as Egypt is concerned, Nixon and President Sadat announced in a joint communique that their two governments will establish a series of working groups to meet in the near future to prepare concrete projects and proposals for bilateral research in the fields of agriculture, "technology, research and development in scientific fields (including space, with special emphasis on the exchange of scientists," and medical research.

Such activities will supplement the present limited scientific cooperation between the two countries, which is funded to the tune of about \$1.5 million a year through excess US currency in Egypt (so-called PL480 funds). At this stage, the proposal is simply an agreement to cooperate and no concrete projects have been decided. Similar arrangements are proposed with Jordan and Saudi Arabia.

As for Israel, the agreement is less specific, referring only to the need for "further encouragement of the fruitful links already existing between the two countries in the scientific and technical field, including space research.

A "NEW ERA" for coal is forecast by the Secretary of State for Energy, Mr Eric Varley, in his introduction to the interim report of the examination into the coal industry (published by the Department of Energy) carried out jointly by the government, the National Coal Board and the mining unions.

After the depression in the British coal industry in the 1960s the report paints a much more hopeful picture of the future for the industry into the 1980s and beyond. Future demand for coal will be at least equal to if not greater than the present 130 million tons a year, predicts the report. But to satisfy this demand the industry will have to "run very fast to keep still" to the tune of £600 million in capital investment by 1985. New capacity of 42 million tons a year will be needed by 1985 to replace the coal lost by exhaustion of existing mines.

Twenty million tons of this is expected to come from new fields. The big Selby coalfield in Yorkshire will contribute up to 10 million tons a year by 1985 (it is hoped) and Mr Varley, in reply to a question in Parliament, said that the possibility of developing the rich seam of coal under Oxfordshire is being actively explored.

## New King Coal

by Eleanor Lawrence

Nine million tons a year would come from the life extension of pits which would otherwise be quickly exhausted, and the remaining 13 million tons from new schemes at existing pits. With open-cast mining a total production of 150 million tons a year is to be the ultimate target.

The upswing in the fortunes of coal follows the dramatic rise in the oil prices during the past year, which has again made coal a fuel competitive with oil. Economic conditions for a thriving industry are right for the first time in twenty years, says Mr Varley. Now the coal industry can be both competitive and commercially self-sufficient.

The electricity boards are the coal industry's main customers, taking 58% of National Coal Board (NCB) production in 1973, and the future of coal depends to a large extent on their long term policies. The Central Electricity Generating Board (CEGB) has told the committee that, providing coal remains competitive with oil, the boards will

continue to take as much NCB coal as they can get. The CEGB reckons that in 1985 it will be able to burn about 90 million tons (compared with the 76 million tons it took in 1973). But the industry fears that this potential demand will not be realised unless new coal-fired generating capacity is built.

In the event the NCB has shown itself amply justified in its whole-hearted endorsement of the future of coal even when things looked blackest. The industry contracted from an output of 210 million tons and a workforce of 770,000 men in 1959 to 130 million tons and 270,000 men in 1973. The government is obviously determined not to return to the situation of the 1960s, when the increasing dependence on cheap oil led to the running down of the industry, and promise in the report that the industry shall not be at the mercy of short term fluctuations in the price of competing fuels.

Mr Varley said in Parliament last week that it would need "superhuman effort" from the industry to meet the ultimate target of 150 million tons a year. Production is down this year and two major strikes in the past three years have cast doubts on the essential condition of security of supply.



## General Atomic's new fusion venture

from David Fishlock

WHILE discussions continue on the design—and location—of JET, the Joint European Tokamak, plans are ready for a start in La Jolla next month on the first of the new generation of thermonuclear fusion machines. These are the machines plasma physicists believe will demonstrate later this decade the feasibility of fusion, by reaching 'break-even'—the point at which the experiment's energy output equals the input.

Dr Tihoro Ohkawa, head of fusion research at General Atomic, the joint nuclear venture between Gulf Oil and Royal Dutch-Shell, has received approval from the United States Atomic Energy Commission for his plans for Doublet III. If all goes well by about the end of 1977 Doublet III will be ready to demonstrate plasma confinement times as long as one second.

Doublet III is the successor to a very successful experiment in magnetic containment called Doublet II, carried out by General Atomic between 1971 and 1973. Dr Ohkawa demonstrated how the plasma itself could be used as an electrical conductor to generate intense magnetic fields that remain unperturbed by the plasma. Doublet III will be a large scale version of this experiment and is expected to cost about \$26 million.

A site has been chosen in the valley below General Atomic's hilltop research campus, so that the power lines supplying some 5 million amperes can conveniently be kept out of sight. The dumpling-shaped structure, some 13 feet tall and 18 feet in diameter, is expected to take 3–5 years to complete. Meanwhile, to assist the designers Doublet II has been stripped down and is now being rebuilt as Doublet IIA, with magnetic facilities for fine tuning the plasma configuration.

In the late 1960s, when the physics of fusion was still in chaos, Dr Ohkawa's fusion research group suffered far more severely than the British Culham Laboratory. After Texan utilities withdrew their support his group numbered no more than seven. But, encouraged by Dr C. L. Rickard, vice-president in charge of advanced systems development, Ohkawa rebuilt confidence among sponsors. Today he can boast the largest fusion team in the private sector anywhere in the world. It numbers 80 and this year will spend about \$7 million. Next year it will number 120.

It draws these funds from three sources—the company, a group of

electrical utility sponsors and the United States Atomic Energy Commission. Of this year's research budget, about \$2 million is being spent on plasma physics, \$4 million on large fusions systems (mostly Doublet IIA) and the remaining \$1 million on technology for the fusion reactor. But this last sector is expected to grow quickly, so that by 1980 it will be at least the size of the other two parts of the programme.

Already a conceptual design is taking shape of a candidate for the prototype fusion reactor which the Atomic Energy Commission hopes to start building in 1976. General Atomic is thinking in terms of a reactor based on Doublet III that will 'piggyback' on the company's experience with helium-cooled fission reactors. Instead of using a liquid-metal-cooled system, as is usually proposed for extracting heat from a fusion reaction, it plans to use helium as the coolant and to drive gas turbines directly with the hot but inactive gas. For the present, therefore, Dr Ohkawa is confining his forays into fusion reactor technology to work on a ceramic torus—of silicon carbide and carbon—in which the plasma will be confined.

## Death-blow dealt to Moscow seminar

from Vera Rich



Slepak: leading activist

INTENSIFIED pressure on the part of the Soviet authorities would seem to have dealt a death-blow to the Moscow 'underground' seminar on "Collective Phenomena and the Applications of Physics to Other Fields of Science" scheduled for July 1–5, 1974. A wave of arrests in the Soviet Union of Jewish dissidents, supposedly in connection with security arrangements for the Nixon visit, coupled with the blocking of visa applications from intending foreign participants, almost certainly mean that the seminar can no longer take place as originally envisaged.

The campaign began, apparently, on Wednesday, June 19, with an attempt to arrest an electronics engineer, Vladimir Slepak, who, although not a member of the Seminar Programme Committee, is a leading Jewish activist in the Soviet Union. Slepak apparently resisted arrest by barricading himself into his apartment; on Friday, however, the police broke in with crowbars. It is reported that when his fifteen-year-old son Leonid attempted to contact western journalists by telephone, the KGB intervened.

On the same day, Committee Member Viktor Brailovskii, in hiding to avoid forcible conscription, failed to keep a prearranged telephone contact with the international organisers of the seminar, while a telephone call to Tanya Levich (the wife of committee member Venyamin Levich) was established and cut off three times so that no message could be conveyed. It now seems that Lerner, Brailovskii and also committee members Mark Azbel, Aleksandr Lunts and Aleksandr Voronel were arrested on Friday or Saturday. Voronel, whose Moscow apartment was to have been the venue of the seminar, was arrested at the home of a friend. He is reported to have "given himself up" to the police—in view of the details of Slepak's arrest, however, this probably means no more than that he opened the door to them. Since all contacts between the dissidents and the outside world have now been broken, it is quite possible that this list of arrests is by no means complete.

Simultaneously with the news of the arrests, the foreign "participants" learned that they themselves would not be able to travel to Moscow. Some 40 scientists from the United States (including seven Nobel Prize winners) have been refused visas. Similar refusals are reported from Israel and France. The British contingent, for whom more details are available, reveal an interesting picture of blocking tactics. Those intending to travel alone were told, on submitting their applications, that they must provide an official invitation from some recognised scientific organisation in the Soviet Union (which, of course, they were unable to do). Since then they have heard no more of their applications. The others, who planned to travel as a group, have not met a definite refusal; but since the last date by which the travel agents needed visa clearance in order to complete the necessary arrangements has now passed, this amounts to *de facto* refusal.

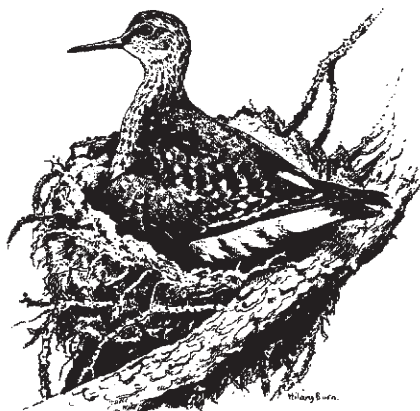
Asked to comment on the situation Dr Norman Chigier of the University of Sheffield, one of the three International Secretaries of the Seminar, observed: "It appears that the Soviet authorities are determined to destroy the seminar".



CITIZENS of the United States are entitled under their constitution to bear arms, and they believe in using them. In addition to shooting public figures and wild game, they are also accustomed to collect any rarities they encounter "for scientific purposes". There is a conspiracy of silence over this in North America, where for example the senior ornithological journal has refused to publish any comments on reports that specimens of the first Little Gulls *Larus minutus* and Wood Sandpipers *Tringa glareola* found nesting in North America were promptly collected despite the fact that they are easily recognised and that there was already ample material available. Even the supposedly militant Audubon Societies have said nothing about the situation. There has, however, been a growing chorus of complaint where the same policies have been followed abroad. Many of the atrocities have been perpetrated by young and inexperienced people, brought up in an unhealthy tradition by their seniors who tend to escape the blame, so it is worthy of note that one Daniel has at least been brought to judgement in his own courts.

For several months now there has been an excited twittering in international ornithological circles as the news went round that Professor Charles Sibley, formerly of Cornell University and now of Yale University, who was awarded the American Ornithologists' Union's Brewster Memorial Award for the electrophoretic analysis of egg-white proteins in 1971 and subsequently elected a Vice-President, has been found participating in the international

## Egg collector collected



Wood Sandpiper: collected

egg trade. It seems that by a skilful piece of deduction Richard Porter of the British Royal Society for the Protection of Birds, concluded that a local egg collector must be dealing with foreign parts, located allegedly incriminating correspondence with Professor Sibley and referred it to the American authorities. Professor Sibley's files at Yale were then inspected, with the result that on May 20 he was fined \$3,000 under the Lacey Act for six cases of "illegally importing bird parts taken abroad in violation of foreign wild life laws", five of them in Britain.

The situation is made worse by the fact that for at least fifteen years the British Home Office Advisory Committees had already been stretching the law to the limit to enable Professor Sibley

to obtain material legally, and many British ornithologists had been assisting him with this. He justified his decision to exceed his allocation by telling the British *Sunday Times* (June 16) that it was an insult to his scientific standing imposed by emotional bird conservation groups with little knowledge of bird population dynamics, which he felt entitled to ignore because "the idea that taking a few eggs could endanger a species is the most ridiculous thing you could imagine . . . I say 'fine, we'll pay the penalty, but you are going to have to listen to the real experts, because you are not the real experts'".

The British Home Office Advisory Committees consist of people of a most unemotional sort who are internationally recognised to have made more important contributions to the study of population dynamics than Professor Sibley. They must have been watching the amount of material that he had already received through legal means, which is known to have been large, rather carefully, and it would be interesting to know where Professor Sibley expects to find greater "experts". It is surprising to find a person in such a responsible position at such a distinguished institution first behaving in such a way and then indulging in such an outburst when he was found out. If senior people behave like this, it is hardly surprising that the conduct of some junior American ornithologists is not all it should be. It is good to see the American authorities take the first steps towards putting their house in order. But there is still bigger game in the woods.—From W. R. P. Bourne.

THE British government has accepted in principle the main recommendations contained in the report of a committee which enquired into causes of the London smallpox outbreak of 1973, when two people died and widespread disruption was caused as vaccination was demanded for four to five millions.

The circumstances leading to the appointment of a committee of enquiry were that a graduate laboratory technician employed at the London School of Hygiene and Tropical Medicine was admitted to hospital on March 16th suffering from suspected meningitis or glandular fever. In fact she had contracted smallpox, and after being placed in an open ward at St. Mary's Hospital, Harrow Road, she came into contact not only with other patients but with two visitors who subsequently contracted the disease and died.

There was serious disquiet that a laboratory worker could have become infected while working in the laboratory; that having become infected, she

## Smallpox report

could spend a week in the general ward while the disease remained undiagnosed; and further, that even after a provisional diagnosis of smallpox, another twelve days elapsed before the visitors were traced, by which time it was too late to save their lives.

The committee found, among other things, that inadequate safety measures were in force at the London School of Hygiene and Tropical Medicine, where the technician was infected while watching the harvesting of pox virus. The pox virus laboratory was said to be old fashioned, poorly decorated, overcrowded and dirty — conditions which, said the report, led to a lowering of safety standards.

Lethal smallpox virus samples were stored in a corridor in an unlocked deep-freeze, workers from other laboratories passed to and fro, using equipment housed in the pox virus lab, and there was an inadequate immunisation

policy in the School's Department of Microbiology. Each unit within the department was left to make its own arrangements in relation to its own hazards.

The report recommended the establishment of a permanent committee to draw up and enforce a code of practice for work on pathogens constituting a major threat to public health; the appointment of a safety officer at the School (already implemented); and, since many of the shortcomings resulted from cash shortages in the first place, financial aid to implement the safety proposals.

Failure to trace contacts in the case were caused by a catalogue of administrative errors, coupled with the fact that no one realised the urgency of the situation, says the report, recommending the appointment of regional specialists to supervise control of outbreaks, consideration of compulsory isolation of contacts, and better diagnostic guides for doctors.

# correspondence

## Unreal Science

SIR,—As a qualified and practising scientist I wish to dissociate myself from your editorial of April 12. You allude to Geller and Velikovsky with terms like “nonsenses”, “beliefs beyond science”, and “pseudo-scientific ideas”, and contrast these with “real science” and “science based on conventional ideas about the way a scientific investigation should proceed”, and you declare that “scientists want to fight this distressing drift from the scientific way of thinking”.

History is littered with ideas shown to be false by people bold enough to question their contemporary conventional science, often in the face of personal ridicule and even persecution.

Presumably you class Einstein's relativity theory with ‘real’ science, yet to back his theory Einstein makes a couple of postulates, not yet proven, whilst the unreal Velikovsky quotes source after source and fact after fact to back his. Where Velikovsky leaps eagerly into the jig-saw puzzle of the past and fits piece after piece together (rather unscientifically perhaps, but incorrectly . . . ?) Einstein treads warily (and of course scientifically) through his mathematical maze, and deigns to emerge into the real world only at the end to explain Mercury's perihelion drift and to predict the deflection of light by the Sun. But wasn't there someone recently who found that the Sun was a little flatter than was previously thought which could account for half that drift (doesn't that make Einstein at least half wrong?), and isn't there someone else suggesting there's a planet, Vulcan, in there somewhere, or even maybe a whole asteroid belt? And just for one careless moment suppose that Velikovsky were right . . . no, of course—much too unscientific!

I am not saying Einstein is wrong and Velikovsky right, but I believe both men's theories worthy of serious consideration. You say “*Nature* has a responsibility (that) guides us in the choice of papers for publication”. You have a right and a duty to edit, but that statement smacks of censorship. Velikovsky encountered censorship. More recently Dingle has met similar problems for daring to question Einstein.

I am not young nor am I by any means a pseudo-scientist, nor are a number of colleagues who share my

opinion. I want no part in any science which operates with a closed mind, and I will encourage the young, and old, to drift from such a way of thinking, though I hardly need to. Your own editorial will do that well enough by itself.

P. WARLOW

Southminster, Essex, UK

## Cancer research

SIR,—Your correspondent Brian Ford comments (*Nature*, May 24) on the abundance of data and paucity of concepts in cancer research. A cursory survey of the literature strongly supports this point.

For example, 10 randomly chosen issues of *Excerpta Medica—Cancer* were found to cite about four thousand papers, of which a total of nine were classified as Theories of Carcinogenesis. It is as though 99.8% of the time taken to solve a jig-saw puzzle went on gathering heaps of pieces and 0.2% went on fitting the pieces together. This imbalance conflicts with the widespread belief that cancer research needs “more facts and fewer theories”. The opposite appears to be the case.

Yours faithfully,

T. E. WHELDON

Department of Clinical Physics and  
Bioengineering,  
Glasgow, UK

## Geology $\supset$ geophysics?

SIR,—The President of the Geological Society of London, the Director of the Institute of Geological Sciences and Professor P. Allen have informed us<sup>1</sup> “that geophysics is a necessary and important part of geology” and in case the reader misses the point they say it again “. . . of earth science, that is of geology.” That a present and past—and one hopes future—president of the oldest Geological Society in the world should so write is doubtless a Freudian slip, as earlier this century the society refused to take geophysics under its wing, this important task being faithfully discharged since then by the Royal Astronomical Society to the mystery of our friends abroad. In the Cambridge Tripos Part II twenty years ago the question was set: “Geophysics dots the i's and crosses the t's of geology. Comment.” I seem to recall that Professor Allen was an examiner. Perhaps those who simply answered

“But, of course” qualified for firsts!

I have always worked, as have your correspondents, for more understanding and dialogue between geophysicists and geologists, but also between geophysicists and astronomers and physicists: problems of the Earth's rotation, Earth and planetary magnetic and gravitational fields, solid state physics of interiors, lunar and planetary formation are of more interest to the latter groups than to geologists. Geologists on both sides of the Atlantic who suppose—perhaps understandably as the highways of earth science are presently congested by careering band-wagons—that continental drift, sea-floor spreading, plate tectonics and plumes is the whole of geophysics might do well to read again Sir Harold Jeffreys's *The Earth* or study Professor A. H. Cook's *Physics of the Earth and Planets*.

As Sir Peter Kent is also Chairman of the Natural Environment Research Council (NERC), it seems desirable to emphasise that geophysics is a discipline in its own right separate from—and one might add older than—geology. The NERC awards of research studentships for the Earth Sciences seem to me to be seriously over-weighted<sup>2</sup> at present in favour of geology as against geophysics by about 7:1. This disparity does not reflect the relative need for highly trained people in these two disciplines nor the present intellectual challenges in the two subjects: it reflects the fact that there are some 45 departments of geology in British Universities.

Thus, as Chairman of the Royal Society committee which launched the European Geophysical Society, I welcome the 1975 “Europe from crust to core” meeting as a further step in European scientific cooperation. I hope that the formation of a ‘Pan-European Society of Earth Scientists’ (as distinct from a European geological society) is not seriously mooted if only on logistic grounds. Geophysicists interested in geological problems will surely come, as I hope to myself, to Reading and geologists concerned especially with tectonics will continue to join us at the European Geophysical Society's annual meetings.

Yours faithfully,

S. K. RUNCORN

University of Newcastle upon Tyne

<sup>1</sup> *Nature*, June 14, 249, 608 (1974).

<sup>2</sup> N.E.R.C. Research Studentships 1974.



# news and views

## Testing for carcinogens and mutagens

It has been known since the work of Pott in the eighteenth century that certain forms of cancer are associated with certain occupations and it is now generally accepted that the disease can be and is caused by chemicals in the environment. The number of chemicals that have been shown experimentally to induce cancer in laboratory animals is very large and constantly increasing but the numbers known with reasonable certainty to be active in human beings is very much smaller. Recognised human carcinogens were listed by Hueper in 1971 (*Health Phys.*, **21**, 689-707; 1971) and included asbestos and some metals, several aromatic amines, various tar and pitch products and mustard gas. Since then adenocarcinomas of the vagina in young women and girls has been linked with exposure to diethylstilboestrol *in utero* following therapeutic administration of the compound to their mothers during pregnancy (Herbst *et al.*, *New Engl. J. Med.*, **284**, 878-881; 1971), the occurrence of benign tumours of the liver has been reported in women taking oral steroidal contraceptives (Baum *et al.*, *Lancet*, **ii**, 926-929; 1973) and, more recently of all, angiosarcoma of the liver has been found in men exposed to vinyl chloride during the manufacture of polyvinylchloride (PVC) one of the most extensively used plastic materials (*Br. Med J.*, **1**, 590-591; 1973).

It is highly significant that these human tumours are all exceedingly rare and therefore the occurrence of even quite small numbers of cases led to the suspicion of an environmental factor in their causation. It is very unlikely indeed that an environmentally-caused increase in a common human tumour would be so readily detected, for example cancer of the lung or colon in the United Kingdom. The realisation that many chemicals are mutagenic has been relatively recent and the problems of detection of altered mutation rates in human populations are formidable. It is therefore of the utmost importance to develop reliable tests for carcinogenicity and mutagenicity of environmental chemicals.

There is substantial agreement on methods for conventional long-term carcinogenicity tests in rodents and these provide the best available means for assessment of possible carcinogenic hazard to man. They are, however, very expensive and require at least 3 years for completion. Methods for mutagenicity testing in mammals are less well established. The specific locus test in the mouse can only be applied to a limited number of chemicals because it requires very large numbers of animals and it is extremely expensive. The dominant-lethal test and cytogenetic analysis *in vivo* require far fewer animals but they are still expensive and time consuming. There is thus a very real need for simpler and less costly tests for carcinogens and mutagens in the environment and much effort is currently being directed towards the development of *in vitro* test systems using cultured cells and microorganisms. Although such tests are unlikely to give conclusive results they may provide valuable information for the assignment of priorities for more definitive test procedures.

The molecular mechanisms of chemical carcinogenesis

are not understood but it is now clear that most of the chemicals that cause cancer (precarcinogens) do so as a result of metabolic conversion into more carcinogenic substances (proximate carcinogens) and finally to chemically reactive electrophilic products (ultimate carcinogens) which react with nucleic acids, proteins and other cellular components and which are also mutagenic (Miller, *Cancer Res.*, **39**, 559-576; 1970). Without assuming the same mechanism for chemical carcinogenesis and mutagenesis it may be concluded therefore that demonstration of mutagenic activity in a chemical should give warning of its possible carcinogenicity.

Apart from *Drosophila*, the organisms commonly used in research on chemical mutagenesis including *Escherichia coli*, *Salmonella typhimurium*, *Neurospora* and *Saccharomyces*, do not contain the mixed function oxidases necessary for activation of precarcinogens and premutagens and do not therefore undergo mutation after exposure to these chemicals. This difficulty has been overcome in the host-mediated assay (Gabridge and Legator, *Proc. Soc. exp. Biol. Med.*, **130**, 831-834; 1969) in which test microorganisms are injected into the peritoneal cavity or elsewhere in mammals, usually rodents, and the putative mutagen is given by another route. The organisms are recovered after an interval and scored for mutants. Several chemicals that are inactive by direct application to the organisms become active in this assay, which makes use of the microsomal or other enzymes of the host. A logical extension of this procedure was to use an *in vitro* system in which the test compound is incubated with mammalian liver microsomes in the presence of the necessary co-factors and suitable indicator microorganisms. For example, dimethylnitrosamine was actively mutagenic when incubated under such conditions with mouse liver microsomes (Malling, *Mutation Res.*, **13**, 425-429; 1971) and human liver preparations have recently been shown to be similarly active (Czygan *et al.*, *J. natn. Cancer Inst.*, **51**, 1761-1764; 1973). Although these *in vitro* methods are relatively easy and quick they still require appreciable time and experimental skill and the process has been simplified further by Ames and his colleagues (*Proc. natn. Acad. Sci. U.S.A.*, **70**, 2281-2285; 1973) who have incorporated a post-mitochondrial liver homogenate into the agar plate used for culture of the test organisms. Using this procedure several chemical carcinogens, mainly planar aromatic compounds, were shown to be frame-shift mutagens. Further work with more and different types of carcinogens is needed before the value of the Ames procedure for large-scale screening of environmental chemicals can be adequately assessed. If it proves sufficiently sensitive and reliable it should, by virtue of its simplicity and cheapness, provide a valuable addition to currently available tests.

More recently Durston and Ames (*Proc. natn. Acad. Sci. U.S.A.*, **71**, 737-741; 1974) have used the combined agar plate-microsome method for the detection of mutagens in urine and the communication by Commoner, Vithayathil and Henry on page 850 of this issue of *Nature*, reports similar experiments. Both groups of workers investigated the urine of rats given the potent carcinogen 2-acetylaminofluorene (AAF) and Commoner *et al.* also studied *p*-dimethylaminoazobenzene (DAB). Both groups found mutagenically active metabolites in the urine of rats treated with AAF and the activity was increased after addition of



$\beta$ -glucuronidase. Commoner *et al.* also found that the urine from DAB-treated animals contained active mutagenic components but only after treatment with  $\beta$ -glucuronidase and take-diastase; this is of interest because Ames *et al.* had previously reported that DAB itself was not mutagenic after exposure to liver homogenates *in vitro*. Clearly many more carcinogens and other compounds must be tested in this system before it can be properly evaluated. Nevertheless,

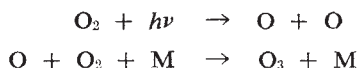
as the authors point out, the test system for urinary mutagens could readily be included in toxicity studies on laboratory animals and it could also be used to screen the urine of human populations for the presence of conjugated and unconjugated mutagens. In this way, the prior ingestion of carcinogens and mutagens in the diet and from other environmental sources might be revealed.

P. N. MAGEE

## Destruction of atmospheric ozone?

THE Earth's atmosphere is extremely effective in filtering out that part of the Sun's ultraviolet radiation which is harmful to life, that is, radiation with wavelengths below about 300 nm. Ultraviolet radiation with shorter wavelengths, below about 200 nm, is absorbed by molecular oxygen,  $O_2$ . The rarefied, but nevertheless strongly absorbing, ozone molecule,  $O_3$ , traps the remaining longer wavelength ultraviolet radiation up to about 290 nm, a region in which  $O_2$  is virtually transparent. The main ozone layer extends very roughly between altitudes of 15–50 km above the Earth's surface.

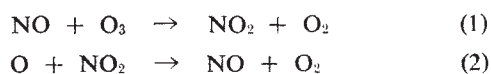
As severe radiation damage to the surface cells of plant and animals is caused by intense ultraviolet light at wavelengths of less than 300 nm, changes in the very low atmospheric ozone concentrations, which filter solar radiation, are of vital concern. Normally, atmospheric ozone concentrations are held in rough balance between the rates of production and destruction of  $O_3$ . Ozone is produced by the photochemical dissociation of  $O_2$ , followed by recombination of the oxygen atoms with  $O_2$  in the presence of a third gas molecule M,



The destruction of  $O_3$  can occur through its reaction with O atoms, or alternatively, by reaction with nitric oxide, NO, which is known to be a trace constituent even of unpolluted atmospheres.

Ozone is a highly reactive molecule, and it has been suggested recently that artificial injection of certain chemical species into the stratosphere may diminish the ambient ozone concentrations by increasing its rate of destruction, thus upsetting the natural ozone balance. The consequently increased flux of far ultraviolet light incident on the Earth's surface would obviously have far reaching, deleterious consequences.

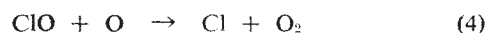
Particular attention has been focused on the possible role of nitric oxide (or equivalent nitrogen oxides, often referred to generally as  $NO_x$ ) in affecting stratospheric ozone. Nitric oxide, which is a minor product of combustion processes, is emitted into the stratosphere by high flying aircraft and by other aerospace vehicles. Johnston, at Berkeley, has proposed the following two-step catalytic cycle for ozone destruction by added nitric oxide:



Thus, reaction (1) and (2) together consume  $O_3$  and O atoms, and provide a mechanism for recycling NO. The rates of reactions (1) and (2) are now well known, and so there is no doubt that sufficient quantities of nitric oxide, if well mixed with the stratospheric atmosphere, could consume ozone in this way. The exact concentrations of the relevant atmospheric species are not well enough known, however, nor are the effects of the local injection

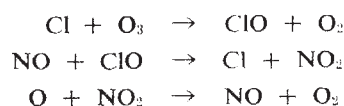
of  $NO_x$ —as opposed to the addition of 'well mixed  $NO_x$ '—clear. There is no general agreement about whether stratospheric ozone levels will be affected by  $NO_x$  injected into the atmosphere by known plans for fleets of supersonic aircraft. Nevertheless, an appreciation of the possibility of a perturbation of the ozone cycle by for example, nitric oxide, is clearly most important. Indeed, concern has been expressed in the British press, following Pittcock's report (*Nature*, **249**, 641; 1974) of a significant downward trend (since June, 1965) in atmospheric ozone concentrations at altitudes up to 25 km above the Earth's surface. It remains to be seen whether or not Pittcock's conclusion, based on carefully executed, but difficult, experiments, is confirmed by other workers. At any rate, there is growing awareness of the importance of the possibility that the natural ozone cycle may be upset by atmospheric pollutants.

That awareness led recently to the discussion of a possible chlorine oxide,  $ClO_x$ , cycle, based on two steps involving chlorine atoms, Cl, and the chlorine monoxide free radical, ClO:



Stolarski and Cicerone, elsewhere, and Molina and Rowland, on page 810 of this issue of *Nature*, consider this cycle, and suggest that Cl atoms are formed in the stratosphere. The suggested methods of formation include the photochemical dissociation of HCl from rocket exhaust gases, and of freons which diffuse upwards from the Earth's surface. Freons, compounds which contain only carbon, chlorine and fluorine, are manufactured extensively as aerosol propellants and refrigerants. Molina and Rowland, working at the University of California at Irvine, consider that the freons  $CF_2Cl_2$  and  $CFCl_3$  have a long residence time in the lower atmosphere. This would allow their concentrations to build up, without any obvious sinks for their removal. Rough calculations suggest that virtually all of the freons ever manufactured now remain in the atmosphere. The upward diffusion of freons, followed by photochemical dissociation, could result in chlorine atoms at stratospheric altitudes.

Watson and I at Queen Mary College, University of London, have determined recently the rates of several of the relevant chemical reactions involving Cl atoms, and  $O_3$  and ClO radicals. Molina and Rowland, using these and other results, point out that, at stratospheric temperatures the attack of ClO on O (reaction (4)) is six times as rapid as that of  $NO_2$  on O (reaction (2)). Consequently, under some conditions, the Cl/ClO cycle can be considerably more efficient than the  $NO/NO_2$  cycle in the catalytic conversion of  $O_3 + O$  to  $O_2 + O_2$ . At some altitudes, a mixed  $NO_x - ClO_x$  cycle may also be important:



Molina and Rowland conclude that the Earth's atmosphere has only a finite capacity for absorbing chlorine atoms without important consequences. According to their view, the present understanding of atmospheric chlorine chemistry suggests that this absorptive capacity may be insufficient even to cope with the number of Cl atoms equivalent to the quantities of freons at present introduced into the atmosphere on a world-wide scale. More accurate estimates of the absorption capacity are desirable, and no account can be taken of possible heterogeneous reaction between Cl atoms and particulate matter in the stratosphere. If the conclusions of Molina and Rowland are correct, then the effects of the stratospheric photodissociation of  $\text{CF}_2\text{Cl}_2$  and  $\text{CFCl}_3$  could not be removed immediately even if no more freons were introduced at ground level. That is because of the postulated long residence time of these species in the lower atmosphere.

The inevitably approximate nature of this type of calculation, and the far reaching assumptions required, should be borne in mind. Discussions such as that of Molina and

Rowland, however, serve a most useful purpose in initiating discussion of a new aspect of atmospheric ozone. It is reassuring that interest in these and related problems is being taken by the US Department of Transportation, in its support of the Climatic Impact Assessment Program, under the auspices of which several valuable discussion documents are being issued.

It is conceivable that sulphur dioxide, emitted in relatively large quantities during the combustion of many natural hydrocarbon oils, such as aviation fuel, might also affect ozone concentrations by the photochemical dissociation of  $\text{SO}_2$  to  $\text{SO} + \text{O}$ , followed by the reaction of SO with  $\text{O}_3$ , which has a rate comparable to that of NO with  $\text{O}_3$ .

This possibility, which requires further consideration, is raised in order to highlight the fragility of the ozone layer and the consequent need for thorough knowledge of the mechanisms and rates of chemical reactions involving ozone.

M. A. A. CLYNE

## Phosphorus and the eutrophication problem

SCHINDLER of the Freshwater Institute, Winnipeg, has described in *Science* (184, 897–898; 1974) the effects on the growth of planktonic algae of fertilising small, unproductive Canadian lakes with compounds of phosphorus, nitrogen and carbon. If nitrogen or carbon were added there was no marked change in the phytoplankton. If phosphorus was added as well or by itself, there were large increases in phytoplankton. When the additions of phosphorus were discontinued the phytoplankton soon returned to the pre-fertilisation levels. Hence Schindler concludes that a large decrease in the phosphorus content of sewage effluents is the essential remedy for the over-enrichment of waters by man or, as it is called, cultural eutrophication. Polyphosphates in detergents should be banned since they form some 50% of sewage phosphorus (somewhat less in Britain). He agrees with those people who advocate the chemical treatment of sewage to remove a substantial amount of all waste phosphorus. But rapid action is needed and the removal of polyphosphates from detergents will meet this need.

Nearly all limnologists who have investigated the problem will agree with Schindler that phosphorus is a major cause of eutrophication. He refers to several lakes which have benefited from the removal of sewage effluents but it is less clear whether the removal of detergent phosphorus alone would have prevented undesirable eutrophication. Some action has been taken. Canada has prohibited the sale of detergents containing more than 2.2% of phosphorus and some American States have passed similar legislation (see Schindler's article); others will remove phosphorus by sewage treatment.

If polyphosphates are to be removed from detergents, in which they play a vital part, what is to replace them? At present there is no generally agreed answer. In Canada nitrilotriacetic acid (NTA) is a permitted alternative. A governmental warning against its use, has, however, been issued in the United States (see statements by Environmental Protection Agency Administrator and Surgeon General (December 18, 1970) and by Chairman, Committee on Public Works, US Senate (December 19, 1970)). As a result substances such as washing soda and sodium silicate have been used and the detergents containing them are said to be of poor quality, as may be expected. Experience of the world-wide use of several organic compounds suggests that extensive and intensive tests are necessary before they are

accepted as safe. Phosphates, though bad for eutrophication are otherwise harmless. One must also realise that any increased cost of a replacement will be passed on to the consumer. It seems therefore that there are arguments for and against Schindler's viewpoint.

European experience supports Schindler's view that phosphates are the commonest major cause of cultural eutrophication and that nitrogenous substances are the next most important cause (Vollenweider, OECD Report DAS/CS1/68.27; 1968). Observations for more than 30 years and experiments with large enclosures in the English Lake District (Lund, *Proc. R. Soc.*, B180, 371–382; 1972; *Verh. int. Verein. theor. angew. Limnol.*, 18, 71–77; 1972 and unpublished data) have also shown that phosphorus is an important factor in eutrophication, though it is an oversimplification to blame everything on one element. The same is likely to be true in other British waters.

The UK Standing Committee on Synthetic Detergents (12th Progress Report, HMSO; 1971) is against the present use of NTA and does not consider that phosphorus removal is as yet justified. Nevertheless its reduction from sewage before it enters the already eutrophic Lough Neagh, Northern Ireland has been advocated (Wood and Gibson, *Water Res.*, 7, 173–187; 1973). Schindler says that the removal of phosphorus from sewage "while fine in principle . . . will take several years to implement to any effective degree, considering the time lags and uncertainty inevitable in financing, planning and constructing such facilities". Sweden and Switzerland, however, have gone ahead vigorously with just such a scheme; other countries are doing so or preparing to do so to various extents. In Sweden, in particular, it seems that an added gain is a general improvement in the final effluent.

The eutrophication problem is not the same everywhere. It is doubtful whether the best remedy for one lake or lake region is the best for all. Phosphate enrichment may produce unwanted changes in one lake but will not do so in other lakes. Moreover, opinions differ about which substances are the most harmful. It seems reasonable that each lake or lake area should be considered separately, bearing in mind the general, though still imperfect, knowledge of eutrophication. Then it can be decided whether remedial measures are necessary, what they might be, whether they are acceptable on economic or other grounds and whether legislation is necessary to implement them.

J. W. G. LUND



## Survival of the Teesdale rarities

from Peter D. Moore  
Plant Ecology Correspondent

RARITY is an attribute which makes an organism peculiarly attractive to mankind, even to biologists. When many rare organisms are gathered together in one place, it is inevitable that biological attention should be focused on that spot. In the British Isles there is no better example of this than the rare plants of Teesdale, especially since they have been threatened and partially obliterated by the development of the Cow Green Reservoir. One good result of this incident is the burst of research activity which it has stimulated, partly as a result of the finance generated by the setting up of the ICI Teesdale Trust Fund, a kind of sad consolation for depleted biological resources.

The major biological problem presented by Teesdale is a phytogeographical one, namely the explanation of the surprising distribution patterns of some of the rarities, such as *Gentiana verna*, *Minuartia stricta*, *Tofieldia pusilla* and *Kobresia simpliciuscula*. In 1956, Pigott (*J. Ecol.*, **44**, 545) presented a detailed survey of the plant communities in Teesdale and suggested possible explanations for the unusual assemblages of species found there. Following the arguments of Godwin (*J. Ecol.*, **37**, 140; 1949), he considered that these communities were relicts of late-glacial vegetation which had survived

because of the poor growth of forest in the area during the postglacial climatic optimum. Factors such as severity of climate and instability of soil may have assisted such survival.

Direct evidence of postglacial survival has been found in the local peat deposits, where plant fragments and pollen grains are preserved (Squires, *Nature*, **229**, 43; 1971 and more recently Turner *et al.*, *Phil. Trans. R. Soc.*, **B265**, 327; 1973). Peat deposits, some dating back to the late-Devensian (last glacial) period, show that light demanding plant species have been present in some quantity throughout the Flandrian (postglacial) period. Woodland cover can never have been complete, therefore, and the refugium hypothesis of Pigott gains support. Furthermore, some of the rare and uncommon plants (such as *Gentiana verna*, *Dryas octopetala*, *Polemonium caeruleum* and *Polygonum viviparum*) have a fairly continuous pollen record, indicative of postglacial survival. Many of the other rare plants, however, cannot be identified to the level of species on the basis of their pollen so that evidence for them is still lacking.

An alternative approach to the problem is the study of the ecophysiology of the rare plants. Arnold (*New Phytol.*, **73**, 333; 1974) has examined the growth of seedlings of two Teesdale plants, *Kobresia simpliciuscula* and *Plantago maritima*. Seeds of these species were germinated at 18° C and the seedlings were grown at 10°, 14° and 18° C at a light intensity of 60 W

m<sup>-2</sup>. Records were kept of leaf length, width and number and net assimilation rate. Both species grew faster in all respects at higher temperatures but, in the case of *Kobresia*, there was a marked increase in growth at 18° C when compared with that at 14° C. Mean summer temperatures in Teesdale are between 10° C and 14° C; temperatures of 18° C were exceeded on only about 3% of summer days. Arnold remarks that *Kobresia* would be favoured by a warmer environment and she finds difficulty in explaining its occurrence at Teesdale in terms of its temperature response. The answer seems to be that temperature response is not an important parameter in this situation. The experiments demonstrate that these species are living suboptimally in Teesdale as far as temperature is concerned, but this is not surprising. The survival of such plants is due to their ability to maintain a positive growth rate where many other more robust species (including trees) fail to do so. The relevance of these temperature response data, if indeed they are relevant, will only be appreciated when further figures are available from the potential competitors of the Teesdale rarities.

## Genes for N<sub>2</sub> fixation on the move again

from a Correspondent

Two years ago Dixon and Postgate reported (*Nature*, **237**, 102; 1972) their success in transferring nitrogen-fixing ability from a N<sub>2</sub>-fixing strain of *Klebsiella pneumoniae* to the common colon bacteria *Escherichia coli*. Greater complications in using plant nodule bacteria from the genus *Rhizobium* as donors of nitrogen-fixing ability were anticipated since the presence of N<sub>2</sub>-fixing activity in *Rhizobium* cannot be shown in laboratory cultures, and the genus *Rhizobium* lacks an effective genetic system for mobilising genes. This difficulty now seems to have been overcome by Dunican and Tierney of University College, Galway (*Biochem. biophys. Res. Commun.*, **57**, 62; 1974) who have been able to transform a derepressed antibiotic resistance transfer factor into *R. trifolii*. This work greatly increases the possibility of mobilising *Rhizobium* genes since this same factor is known to mobilise with an appreciable frequency genes in other bacterial systems. With this R factor present the *Rhizobium* acted as if it had had a sex factor and transferred the ability to fix nitrogen when mated with a non-fixing strain, *Klebsiella aerogenes*. Hybrids capable of fixing nitrogen, measured by the acetylene reduction technique, oc-



Aerial view of Upper Teesdale (looking north-west) showing the Cow Green area before it was flooded for the reservoir built by the Tees Valley and Cleveland Water Board (Imperial Chemical Industries photograph).

urred with a frequency of  $10^{-7}$  or higher. Some of these colonies retained the ability to fix nitrogen when subcultured and in these cases maintenance of the R factor was not essential for retention of  $N_2$ -fixing ability. Not unexpectedly, the  $N_2$ -fixing *Klebsiella* hybrids acted like most naturally-occurring free-living  $N_2$  fixers in fixing nitrogen only under anaerobic conditions and in the absence of combined nitrogen.

The ability of *Rhizobium* strains to fix nitrogen only when within nodules on the roots of legumes has puzzled scientists for more than half a century. These bacteria grow in culture media but have never been shown to fix nitrogen *in vitro*, a fact which has in the past often prompted the suggestion that the legume plant contributes at least part of the genes concerned with fixation. Dunican and Tierney suggest that the  $N_2$ -fixing ability is present though not phenotypically expressed in *R. trifolii* and this ability is expressed when transferred to a new bacterial host. The alternative explanation that genetic material transferred from the *Rhizobium* activates certain latent  $N_2$ -fixing genes in *K. aerogenes* cannot be entirely ruled out.

It is anticipated that this genetic system may be useful in studies of the physiology of legume root nodules and may also provide a means of improving strains of *Rhizobium* used in agriculture as seed inoculants. Legumes such as soybeans fix only about one quarter of their nitrogen requirements and a significant improvement in field fixation rates may be possible.

## Radius of neutron distribution in lead

from Peter E. Hodgson

Nuclear Theory Correspondent

DURING the past few years there has been great interest in determining the sizes of atomic nuclei, and in particular the relative spatial extents of the constituent neutron and proton distributions. The proton distributions can be determined accurately from analyses of electron elastic scattering and muonic X rays, but the neutron distributions can only be determined indirectly, and the results are less accurate due to the lack of knowledge of the nucleon-nucleon interaction. Many different methods have been used, and the results are in substantial agreement.

On the whole it is found that the proton and neutron distributions are quite similar, with perhaps a slight tendency for the neutron distribution to extend further from the centre of the nucleus than the proton distribution, particularly for heavier nuclei. There is also the possibility of a 'neutron halo', a

very diffuse neutron cloud extending far beyond the nuclear surface, where protons cannot reach due to the Coulomb field.

A quantity of particular interest is the ratio of the root mean square neutron and proton radii, as this is a convenient way of specifying the relative extents of the neutron and proton distributions. Recent work has tended to give a value of about 1.05 for this ratio though some studies have given values below unity.

A recent measurement of this quantity has used the (p,n) reaction to the isobaric analogue state of the target nucleus. This reaction concerns principally the outer neutrons and protons, and so is particularly sensitive to their densities in the surface of the nucleus. The final state has the same structure as the initial state and differs from it only by the substitution of a proton for a neutron in the highest quantum state. Another way of describing this is to say that the final state is formed from the initial state just by an isospin flip that changes a neutron into a proton. The interaction is thus brought about only by the isospin component of the nucleon-nucleon interaction, which determines the isospin term in the nucleon optical potential. Also affecting the isospin potential is the difference between the neutron and proton density distributions, so the reaction is sensitive to just the physical quantities of interest. All the interactions responsible for the (p,n) reaction are quite well known, and so is the proton distribution, so comparison between the experimental data and a series of neutron density distributions enables the latter to be determined.

The dependence of the cross section of these quantities is particularly simple if the distorted wave Born approximation is used. Then the cross section is proportional to the square of the transition amplitude

$$T(p,n) = [2(N-Z)^{1/2} / A] \int \chi_n^-(r) U_i(r) \chi_p^+(r) dr$$

where  $\chi_n^-(r)$  and  $\chi_p^+(r)$  are the waves describing the outgoing neutron and incoming proton respectively and the isospin potential

$$U_i(r) = [A / (N-Z)] \int u_i(|r-r'|) \{ \rho_n(r') - \rho_p(r') \} dr'$$

where  $u_i(|r-r'|)$  is the isospin component of the nucleon-nucleon interaction and  $\rho_n(r')$  and  $\rho_p(r')$  are the neutron and proton density distributions respectively.

Schery, Lind and Zafiratos (*Phys. Rev.*, **C9**, 416; 1974) have measured the cross section for the (p,n) reaction to the isobaric analogue state of  $^{208}\text{Pb}$  at 25.8 MeV. They calculated the cross section from the above formulae using standard values of  $u_i(|r-r'|)$  and the optical potentials that fix the distorted waves  $\chi_n^-(r)$  and  $\chi_p^+(r)$ . They assumed that the neutron density distribution has the form

$$\rho_n(r) = [1 + \exp\{(r-R)/a\}]^{-1}$$

and varied  $R$  and  $a$  to optimise the fit to the measured (p,n) cross section. The

best fit accounted well for the angular variation of the crosssection, which confirms the reliability of the model. The optimum values of the parameters  $R$  and  $a$  correspond to a ratio of the root mean square radii  $\langle r_n^2 \rangle^{1/2} / \langle r_p^2 \rangle^{1/2} = 1.07 \pm 0.03$ , thus confirming previous work indicating a slightly larger neutron distribution. Since  $\langle r_p^2 \rangle^{1/2}$  is 5.30 fm, the value of  $\langle r_n^2 \rangle^{1/2}$  is 5.68 fm.

The stability of this result with respect to small changes in the assumed potentials was investigated, and this accounts for part of the suggested 3% uncertainty. Studies of the effect of the difference between the unbound proton wave function in the final state of  $^{208}\text{Bi}$  compared with the corresponding bound neutron wave function in  $^{208}\text{Pb}$  showed that the longer tail of the former reduces the ratio of root mean square radii by about 1%. The effects of two step contributions to the reaction was also studied and found to be small.

## Plant messenger RNA and poly(A)

from our

Plant Cell Physiology Correspondent

THE discovery that many animal messenger RNA molecules contain regions of poly(adenylic) acid is of considerable interest (see *Nature*, **247**, 426 and 512; 1974). One reason for this is that the presence of poly(A) confers specific properties on mRNA molecules which can be exploited during RNA purification. Many workers have used columns of oligo(dT) cellulose or poly(U) sepharose which retain the mRNA by specifically base pairing with the poly(A) region. Another approach has been to use unmodified cellulose or nitrocellulose to purify the mRNA.

A recent report shows that presumptive mRNA from plants, long known for its high content of adenylic acid, contains poly(A) tracts (Higgins *et al.*, *Nature new Biol.*, **246**, 68; 1973). Sagher *et al.* (*Biochim. biophys. Acta*, **349**, 32; 1974) now suggest that the length of poly(A) in higher plants is of the order of 150–250 nucleotides which contrasts with the 50 or so residues found attached to yeast and fungal mRNA (McLaughlin *et al.*, *J. biol. Chem.*, **248**, 1466; 1973; Reed and Wintersberger, *FEBS Lett.*, **32**, 213; 1973; Silver and Horgen, *Nature*, **249**, 252; 1974). At present only the poly(A)-containing RNA from polyribosomes of plants which synthesise a large number of proteins has been analysed and not surprisingly it is heterodisperse. Individual mRNAs of known function have yet to be isolated but exploitation of the poly(A) tag may speed up the process. Although this approach to the study of mRNA metabolism holds out great promise it should not be accepted



without reservations. For example, it is by no means certain what proportion of mRNAs contain poly(A). In animals only the histone mRNAs have been shown to lack poly(A) but others may be added to this list and it is too early to assume that all plant mRNAs contain poly(A). It is also probably true that some nuclear RNA molecules that contain poly(A) do not function as mRNAs.

The most favourable situations for the isolation of a single mRNA exist where cells devote a large part of their protein synthesis to the production of one or a small number of specific proteins. One can anticipate that already in a number of laboratories there is a search under way for the mRNAs for the storage proteins of legume cotyledons and the larger and smaller subunits of the fraction I protein.

## Nuclear magnetic order in solid $^3\text{He}$

from a Correspondent

THE long predicted nuclear magnetic ordering transition in solid  $^3\text{He}$  seems to have been observed at a temperature of 1.17 mK in an experiment carried out at Cornell University by Halperin, Archie, Rasmussen, Burhman and Richardson, and reported in a recent issue of *Physical Review Letters* (32, 927; 1974).

The compressional cooling technique used in this work, which has been developed during the past few years by the low temperature group at Cornell, has already been extraordinarily fruitful in that it has led to discovery of the two new and apparently superfluid states of liquid  $^3\text{He}$ . The technique, known as Pomeranchuk cooling after the Russian physicist who originally suggested it, is based on the fact that for temperatures below about 0.3 K,  $^3\text{He}$  has a negative latent heat of solidification so that, unlike other materials, it absorbs heat while changing phase from liquid to solid.

In practice, after being precooled to about 15 mK by means of a dilution refrigerator, the liquid is then steadily compressed by the application of external pressure. At a pressure of about 34 atmospheres it starts to solidify and, because heat is thus being absorbed, it also starts to cool. The cooling effect can be maintained until, eventually, the cell is entirely filled with solid. The final temperature depends on a number of factors including the starting temperature, the rate at which heat leaks into the system or is developed by mechanical inefficiencies in the compression mechanism itself, and the rate at which the liquid is compressed, but is usually in the range 1–2 mK. The  $^3\text{He}$ , which is the subject of the present

experiments, is thus able to act as its own refrigerant from which accrues the enormous benefit that the usual formidable problems of thermal transfer and equilibrium in the low mK temperature range are automatically minimised.

Unlike  $^4\text{He}$ , the  $^3\text{He}$  nucleus possesses a magnetic dipole moment and it is to be expected that, at some sufficiently low temperature  $T_c$ , a transition will occur in which the nuclei become aligned in some ordered fashion relative to each other. Because the nuclear moments are so tiny the ordering temperature is bound to be exceedingly small and, just considering the direct magnetic interaction of the nuclei, it may be calculated that  $T_c \sim 10^{-6}$  K. More refined calculations, however, taking account of the so-called exchange forces, suggest  $T_c \sim 10^{-3}$  K, which is just within the experimental range made accessible by Pomeranchuk cooling.

In essence the basic Cornell experiment was extremely simple: the volume occupied by the  $^3\text{He}$  was reduced at a steady rate while its pressure was continuously monitored. The pressure, with a mixture of liquid and solid in the cell, was necessarily always that of the melting curve at the relevant temperature and, since the melting pressure increases with falling temperature, the pressure automatically gave a measure of the temperature. Under these conditions a plot of pressure  $P$  against  $t$  is almost linear, corresponding to cooling of the sample at a constant rate, provided that no phase changes occurred in either liquid or solid apart, of course, from the continuous solidification-action process responsible for the refrigeration. In practice, the original experiment showed one change of slope and one discontinuity in the  $P(t)$  plot, and these were eventually proved to be caused by two superfluid transitions in the liquid phase. In the present work a third anomaly in the  $P(t)$  graph was observed at the even lower temperature of 1.17 mK.

The interpretation of these experiments, where both liquid and solid are simultaneously present, is of course extremely difficult. Through a series of ingenious additional experiments, however, in which heat pulses were applied to the helium while compression was continued at a steady rate, the authors seem to have demonstrated unambiguously that the  $P(t)$  anomaly at 1.17 mK corresponds to a phase change within the solid, which must presumably represent the onset of magnetic ordering, and they have been able to deduce that the entropy of the solid falls by a factor of 5 within a temperature range of only 100  $\mu\text{K}$ .

It has not been possible from these experiments to deduce the nature of the magnetic order below  $T_c$ . Measurements of the nuclear susceptibility at higher

temperatures had led to the development of a detailed model which predicted a transition at 2.2 mK to an antiferromagnetic state, in which neighbouring nuclei point in opposite directions. The fact that the actual transition has now been found to occur at only half this temperature is in itself strong evidence of the inadequacy of this model. A great deal of further work will certainly be required before a proper understanding of magnetic ordering in solid  $^3\text{He}$  can be attained, but it is very encouraging to know that the phenomenon occurs in a temperature range which has now become accessible to experiment.

## Chemical heat at oceanic ridges

from Peter J. Smith  
*Geomagnetism Correspondent*

NEAR the crests of active oceanic ridges the mean heat flow is generally higher than the global average, and measurements are considerably more scattered than those in, for example, typical ocean basins. Both of these observations are explicable in general terms, for hotter mantle-derived material rises beneath ridge crests to form new ocean floor, and a combination of rugged ridge topography and uneven sediment cover tends to produce both high measurement errors and a distortion of the heat flow pattern (for example, by thermal refraction). Nevertheless, in view of the importance of testing the seafloor spreading hypothesis as rigorously as possible, such loose explanations are not entirely satisfactory; and it is not unreasonable to suspect that the rather messy heat flow picture may be concealing important information about physical and chemical processes in mid-ocean regions.

Because of the large scatter of data, for example, there may be legitimate doubt about the validity of the mean heat flow values themselves, all the more so as McKenzie (*J. geophys. Res.*, 72, 6261; 1967) has shown that they are inconsistent with the formation of new oceanic crust at a reasonable temperature. Mean heat flow as measured over ridge crests may indeed be high, but it is not as high as might be reasonably expected.

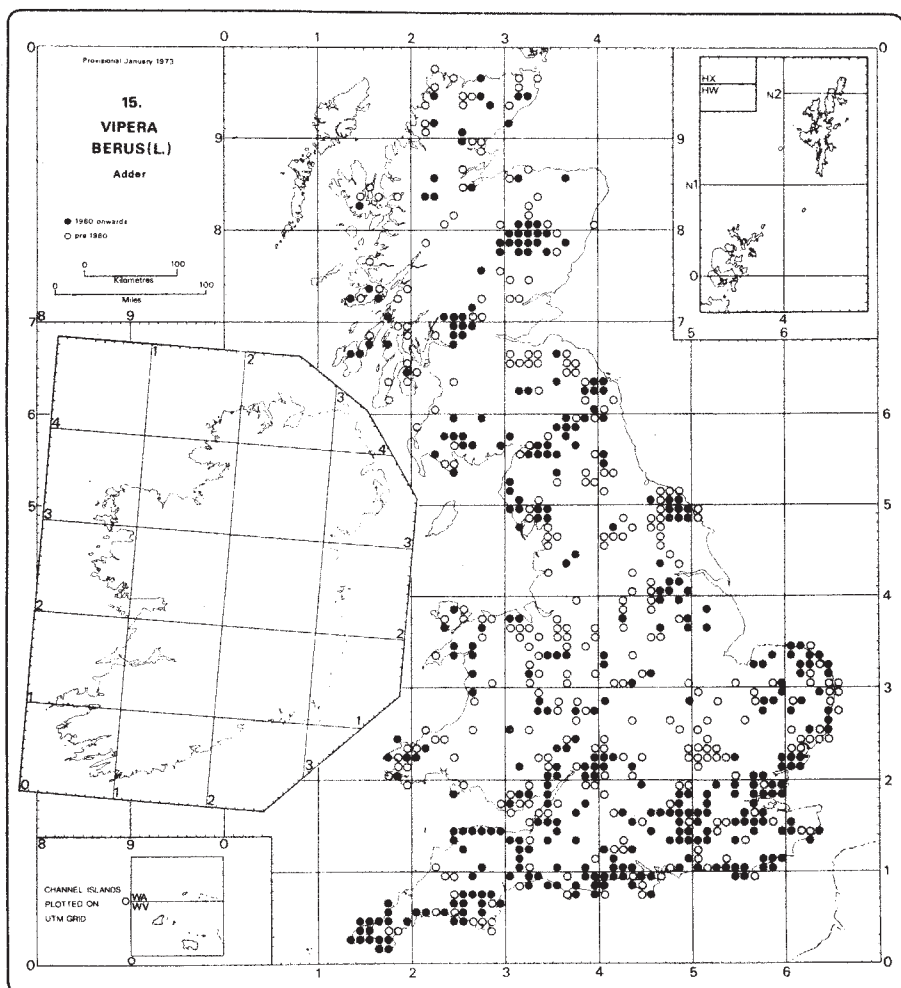
Recognising the scope for investigation here, Lister (*Geophys. J.*, 26, 515; 1972) made an important series of heat flow measurements on the Juan de Fuca ridge and near the Explorer trough in which he took care to assess topographic effects and the thermal influence of recent sediment disturbances. The conclusion he came to was that the thermal balance of a ridge crest zone can be interpreted in terms of a

simple model of seafloor spreading, but only if a considerable part of the surface heat loss at the crest is non-conductive. He thus proposed that in addition to conduction, a significant proportion of crestal heat is carried away by convection in the form of open hydrothermal circulation within the upper 5–10 km of the rock material at the ridge crest. The critical requirement here is that the oceanic crustal rocks should be permeable enough for the circulation to take place; and although relevant data are sparse, there seems little doubt that this is the case. Accepting hydrothermal flow, therefore, it follows that the (conducted) heat measured will be less than the total heat actually generated, thus explaining the discrepancy noted by McKenzie.

The existence of hydrothermal circulation may also explain other things, including the long-time puzzle of why anomalously low heat flow is commonly found on ridge flanks. Moreover, in a rather different field, and as Spooner and Fyfe (*Contr. Mineral. Petrol.*, **42**, 287; 1973) have discussed at some length, "vigorous convective circulation of brine within the oceanic crust" seems to be the phenomenon most likely to account for the pattern of metamorphic rocks found on the ocean floors. In other words, hydrothermal flow is "a significant process at axes of seafloor spreading". Just how significant it is in thermal terms is indicated by a simple calculation. One cubic kilometre of liquid basalt at 1,200° C could heat 3 km<sup>3</sup> of sea water to 300° C (lower than the boiling point at a depth of 1 km under a hydrostatic pressure gradient). This means that at the present rate of magma production all oceanic water could be so heated during circulation through oceanic crust in about 100 million years. As Spooner and Fyfe pointed out, all this suggests that convection within the crust constitutes the major process by which the cooling of the intrusive material in the crust takes place.

But Fyfe (*Geophys. J.*, **37**, 213; 1974) has now drawn attention to another implication of hydrothermal flow. The chemical processes induced by such circulation are oxidation, hydration, carbonation and the fixation of sulphur, all of which are exothermic reactions. Thus magmatic heat induces large-scale convection and the convection then induces further heat-producing reactions. To estimate just how much heat is likely to be generated in this way, Fyfe assumes that the upper 15 km of the oceanic crust and hydration of basaltic pyroxene (a thicker section than Lister suggested, to "allow for some tectonic mixing of the surface layers as the conveyor belt

## Atlas of British amphibians and reptiles



THE number of adders (*Vipera berus*) present in the British Isles seems to be reasonably healthy; as this distribution map shows, records of sightings have been returned from most parts—except, of course, from Ireland. As the new *Provisional Atlas of the Amphibians and Reptiles of the British Isles* (edit. by Arnold, Biological Record Centre, 25p) reports, however, three of the remaining 11 indigenous species—the natterjack toad (*Bufo calamita*), sand lizard (*Lacerta agilis*) and smooth snake (*Coronella austriaca*)—are now extremely uncommon and will need protection if they are going to survive. Staff at the Biological Records Centre at Monks Wood Experimental Station, Abbots Ripton, are aware of the provisional nature of the atlas; there are several areas where no recording of the commoner species has been done and they will be pleased to receive any record that fills in a gap on one of the maps. The atlas includes brief notes on the identification of species.

moves") and that the crust comprises 10 km of basalt and 5 km of peridotite. He then calculates that, in a 1 cm<sup>2</sup> column, serpentinisation of the peridotite would produce 10<sup>8</sup> cal, oxidation of 10% FeO in basalt would give 2 × 10<sup>8</sup> cal, hydration and addition of CO<sub>2</sub> to basaltic anorthite would yield 2 × 10<sup>8</sup> cal and hydration of basaltic pyroxene would give 10<sup>8</sup> cal. This gives a total heat source of about 6 × 10<sup>8</sup> cal—comparable to the radioactive heat generated in an equivalent column of

granite over a period of 20 Myr.

Insofar as the chemical reactions considered by Fyfe are probably simpler than those actually taking place, the accuracy of this estimate of heat production is difficult to assess. Nevertheless, it is obvious that there is a potential heat source here which is far from trivial. Fyfe contends, therefore, that such chemically generated heat can hardly be ignored in considering the thermal regime of the ocean floor and oceanic ridges.



# Solar geomagnetic disturbances and weather

by John Gribbin

ONE of the key links in the developing theory that atmospheric circulation (and hence climate) on Earth is affected by solar activity has been provided by evidence that geomagnetic activity can be connected with a deepening of troughs in the atmosphere. But although evidence presented by Roberts and colleagues (see *J. atmos. Sci.*, **30**, 135–140; 1973) has seemed to show that troughs at the 300 mbar level are affected in this way, and although other groups have presented evidence that even surface pressure responds to solar geomagnetic disturbances (see Mustel, *Soviet astr.*, **10**, 288–294; 1966) the reality of the claimed effect has been queried by others. In particular, Stolov and Shapiro found no evidence to support the claim that atmospheric circulation is disrupted during disturbed geomagnetic periods, according to a paper published three years ago (*Proc. Symposium on Solar Corpuscular Effects in the Troposphere and Stratosphere*, Moscow, 1971). So the recent publication of another paper by the same two authors, in which they report that a re-analysis of the data does show such an effect, is particularly important (*J. geophys. Res.*, **79**, 2161–2170; 1974).

The reason for the emergence of the effect from the new analysis seems to be that the strong seasonal trend in 700 mbar heights which is introduced by ordinary meteorological factors must be removed before the solar geomagnetic effect can be picked out from the data. The data themselves are comprehensive, covering the period January 1, 1947 to December 31, 1970. They consist of sea level pressures and 700 mbar heights measured at 5° latitude intervals and 10° longitude intervals in a diamond shaped grid over 20° to 70° N. The solar geomagnetic incidents studied in the analysis were chosen from a variety of sources which gave a total of 454 events, some of which were related to one another.

Using a superposed epoch analysis and removing the seasonal effects which dominate the atmospheric variations, Stolov and Shapiro find “firm statistical evidence . . . that strongly suggests the existence of a real relationship between solar geomagnetic storms and the subsequent behaviour of the 700 mbar contour height”. The effect is more pronounced in winter, when the zonal flow is increased 4 days following geomagnetic disturbance. In summer, a smaller but still significant effect occurs 2 days

after such disturbances. At its maximum (for the quadrant 90° to 175° W in winter) the effect corresponds to a 7% increase in the mean westerly flow. Stolov and Shapiro say that “no acceptable physical observations are available to explain the observations”; however, that no longer seems to be strictly true in the light of King’s recent investigation of links between the magnetosphere and atmosphere (*Nature*, **247**, 131–134; 1974). What is certainly true is that further statistical studies are needed, and that these might contribute both to weather forecasting and to an understanding of climatic change.

## Vole populations and their diet

from our Animal Ecology Correspondent

ONE of the greatest drawbacks to the theories which purport to explain the factors causing vole population cycles is their relative untestability (for example, Chitty, *Can. J. Zool.*, **38**, 99; 1960; Christian and Davis, *J. Mammal.*, **47**, 1; 1966) and recent attempts to elucidate behavioural or genetic factors have been inconclusive. Batzli and Pitelka (*Ecology*, **51**, 1027; 1970) and Schultz (*Symp. Brit. Ecol. Soc.*, 57; 1964) have suggested that nutritional aspects of forage may play an important part in regulating cycles. These could take the form of potassium or phosphorous deficiencies which bring about specific or general debility.

Another nutritional factor which might be involved is the toxicity of many food species of plant (Freeland, *Am. Nat.*, **108**, 238; 1974). From the huge mass of published work on species preferences of vole food at various stages of the population cycle, Freeland argues that the loss of animals from peak population densities is the direct result of reduced viability induced by the consumption of plants, or parts of plants, that contain toxins. It is known that quite small amounts of toxins imbibed by voles cause inhibition of protein synthesis and growth, cessation of the reproductive processes, occurrence of megalocytes in the liver together with haemorrhagic necrosis, hypertrophy of the spleen, and cause neural and digestive disorders. If a vole eats more than one toxic species the effect of the toxins they contain may be mutually enhanced.

Freeland puts forward a hypothesis with four central tenets: (1) voles must have a preference for non-toxic species; (2) with increasing population numbers these preferred foods should become scarcer with a concurrent increase in availability of non-preferred, toxic species; (3) at high population densities voles ingest quite large quantities of toxic foods leading to a reduction in

growth and a slowing of reproduction; and (4) in the absence of grazing, the preferred non-toxic species should compete more strongly for space than the less preferred toxic species. The replenishment of favoured food so produced following a ‘crash’ of population allows another cycle to start.

In 1965 Thompson (*Amer. Midl. Natur.*, **74**, 75) examined the toxicity level of many food species of the voles *Microtus pennsylvanicus* and *M. californicus* in relation to their preferences of selection. A plant was ranked as toxic only if it was known to contain a compound or compounds that cause death or severe physiological damage. Bitter tasting or foul smelling species were not rated as toxic unless a truly toxic compound occurred as well. The result of Thompson’s laboratory trials was that voles prefer non-toxic plants like *Trifolium repens* and *Lolium multiflora* to toxic ones like *Ranunculus* and *Rumex*. Batzli and Pitelka have found that during one cycle of *Microtus* abundance the amount of toxic species available becomes relatively more abundant in the face of a diminishing supply of preferred species. The establishment of exclosures indicated that non-toxic species out-compete toxic ones. In areas grazed by voles there is a reciprocal fluctuation of the relative abundances of these two species types.

Krebs *et al.* (*Ecology*, **50**, 587; 1969) have noted that during population decline the rates of disappearance of different age and sex classes of voles are not the same. There is evidence that male sex hormone increases the activity of liver microsomal enzymes which bring about the degradation of toxins (Parke, *The Biochemistry of Foreign Compounds*, Pergamon, Oxford; 1968). So the differential mortality rates frequently observed by field workers is a phenomenon not incompatible with Freeland’s hypothesis.

The crux of the hypothesis is that numerical change must come about sufficiently slowly for there to be a compositional change in the vegetation. Even though *Microtus* and similar rodent species have a high capacity for population increase, natural wastage to predation keeps the numbers low enough to avoid a hasty overgrazing of the vegetation. The slow build-up of rodent numbers produces a slow increase in availability of toxic species which, in time, decreases rodent abundance and allows the whole cycle to start again.

This hypothesis has merit and warrants close scrutiny. At the same time could population ecologists consider not so much how the cycle works as much as why it happens at all? Are cyclical rodents really in such a bad state of balance with their ecosystems, or is there another reason?

# How history has blended

H. V. Wyatt

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*The experiments of Hershey and Chase, giving evidence that DNA is the genetic material, have been greatly simplified and modified in subsequent descriptions. Students are in danger of receiving a quite misleading impression of the process of scientific discovery.*

It was the classic paper of Hershey and Chase<sup>1</sup> which marked the psychological watershed after which DNA was rapidly accepted as the genetic material of living organisms. Looking back, it is difficult to see why this work was accepted whereas Avery's demonstration that transformation was effected by DNA took longer to be appreciated (see for example refs 2–5). Moreover, whereas Avery's 1944 paper<sup>6</sup> still holds, the conclusions of Hershey and Chase have since been modified in detail.

The tightly constructed and interlocking experiments of Hershey and Chase have in most cases been reduced to only one experiment—the Blender experiment—which can be vividly portrayed by diagrams and is therefore popular in the textbooks. In the process of canonisation, their experiments are remembered 'with advantages'.

In this account, I attempt to review the Hershey and Chase experiments in the context of the discovery of transformation and the identification of the genetic role of DNA and to show how they have since been admitted to the Pantheon.

In their paper<sup>1</sup>, Hershey and Chase describe the experiments in the following order. (1) Plasmolysis produces phage ghosts of protein and releases DNA into solution. (2) Phage DNA becomes sensitive to DNase after adsorption to heat-killed bacteria. (3) Phage DNA is liberated when phage adsorbs to bacterial fragments. (4) Removal of phage coats from infected bacteria (the Blender experiment). (5) Only 1% of the sulphur from the infecting phage is transferred to the progeny (including a modified Blender experiment). (6) Thirty per cent of the phosphorus from infecting phage is transferred to the progeny. (7) Formalin-inactivated phage can attach to, but cannot inject their DNA into bacteria.

The experiments formed a logical sequence. The first three provided further evidence for the functional differentiation of the protein coat from the enclosed DNA and continued the work of Herriott<sup>7</sup> and Northrop<sup>8</sup>. The Blender experiment (a term used by Hershey<sup>9</sup>) showed this elegantly. Experiments 5 and 6 were altogether different and showed differential transfer of protein and DNA to phage progeny. The most important experiments, 4 and 5–6 are therefore complementary: alone, none is convincing. The two sets of experiments originated from two sources<sup>2,9</sup>. The Blender experiment followed from Herriott's idea<sup>7</sup> of a microsyringe the genesis of which lay in Avery's work and that of Hotchkiss. The progeny experiments followed those of Watson and Maaløe<sup>10</sup>.

The Blender experiment has since achieved so much fame that it is almost the only part of the Hershey and Chase paper which is still quoted. The novel use of the Waring Blender has perhaps stirred the imagination. Davis *et al.*<sup>11</sup> for instance give an account of experiment 2 and of the Blender experiment, adding that these showed that "the phage DNA carries the genetic information and that infection consists of the penetration of the viral DNA into the cells". The results of the Blender experiment varied throughout the paper (Table 1), doubtless because a number of different experiments were reported in

different contexts. The experiments showed that 65 to 85% of the <sup>32</sup>P and 25 to 18% of the <sup>35</sup>S was not stripped from the bacteria. Hershey and Chase inferred from both types of experiment only that the protein coat "probably has no function in the growth of intracellular phage. The DNA has some function." They added "Further chemical inferences should not be drawn." In the discussion they stated that the "following questions remain unanswered. (1) Does any sulphur-free phage material other than DNA enter the cell? (2) If so, is it transferred to the phage progeny? (3) Is the transfer of P to progeny direct or indirect?" At the Cold Spring Symposium, Hershey<sup>9</sup> spelt out the limitations of the Blender experiment. (1) Does the 20% of the virus DNA which is strippable mean that some of the DNA of the virus is dispensable or that some fails to inject? (2) Does the 20% of the protein that is not strippable have any function in the growth of the intracellular virus? (3) Does the non-strippable protein consist of the stubs of the tails only, or is some protein injected along with the DNA?

## Acceptance of the Blender experiment

Mirsky suggested<sup>12</sup> that the purity of DNA—the transforming principle—might include 2 to 3% protein even in a so-called pure preparation. It was this degree of contamination which made it seem possible that protein might still be the genetic carrier because Mirsky calculated that transformation required about 10<sup>9</sup> molecules of transforming principle.

Hotchkiss had meanwhile shown that the amino acid recovered from acid hydrolysates of purified transforming principle was glycine, arising from hydrolysed adenine—not from contaminating protein. This work was originally published in France<sup>13</sup> and virtually lost<sup>14</sup>. Hotchkiss, however, later gave it at a symposium attended mainly by biochemists and probably only Herriott from the phage groups<sup>2</sup>; the contamination of transforming DNA by protein was less than 0.02%, if at all<sup>15</sup>.

Yet the contamination in the Blender experiment was of the order of 25%; why was this ignored by those who accepted the phage work? First, in the Blender experiment, almost all the infected bacteria survived the treatment and produced infectious phage and almost all had been infected by only one phage (ratio of phage to bacteria was 0.28). The contamination of protein (<sup>35</sup>S) in the Blender experiment was shown in a non-blender experiment to be associated with the bacterial cell wall and to be mainly coats of parental phage particles, presumably identical with the coats which could be sheared off. Hershey and Chase concluded that this protein probably never entered

Table 1 Published data of the Blender experiment

|                   | <sup>32</sup> P | <sup>35</sup> S |
|-------------------|-----------------|-----------------|
| Hershey and Chase |                 |                 |
| Table 5           | 21–24           | 81–82           |
| Figure 1          | 35†             | 80†             |
| Text on page 48   | 21–35           | 75–80           |
| Discussion        | (“most”)‡       | at least 80     |
| Summary           | 15              | 75              |
| Hershey (1953)    | 20              | 80              |
| Limits of figures | 15–35           | 75–82           |

\*Phage material stripped from infected bacteria.

†My calculations.

‡Item in bracket refers to isotope not eluted.



**Table 2** How the Hershey and Chase experiments have been cited

|                                     | <sup>32</sup> P                         | % Isotope eluted* | <sup>35</sup> S                              |
|-------------------------------------|-----------------------------------------|-------------------|----------------------------------------------|
| Garen and Kozloff <sup>31</sup>     | 20                                      |                   | 80                                           |
| Stent <sup>22</sup>                 | < 20                                    |                   | > 80                                         |
| Herskowitz <sup>24</sup>            | 0                                       |                   | 97                                           |
| Cohen <sup>32</sup>                 | 20                                      |                   | > 80                                         |
| Beadle and Beadle <sup>33</sup>     | very little                             |                   | { viral coats<br>contained <sup>35</sup> S } |
| Hershey <sup>34</sup>               | (most with cells)                       |                   | most                                         |
| Hotchkiss <sup>14</sup>             | 15 or less                              |                   | 80                                           |
| Clowes <sup>25</sup>                | (most transferred)                      |                   | mostly                                       |
| Fraser <sup>26</sup>                | little                                  |                   | 70                                           |
| Davis <i>et al.</i> <sup>11</sup>   | { essentially all<br>remained in cell } |                   | 80                                           |
| Hayes <sup>21</sup>                 | 20                                      |                   | 80                                           |
| Pollack <sup>30</sup>               |                                         |                   | > 99 protein                                 |
| Stent <sup>5</sup>                  | 20                                      |                   | 90                                           |
| Stent <sup>23</sup>                 | 20–35                                   |                   | 75–80                                        |
| Stent <sup>35</sup>                 | 20 or less                              |                   | at least 80                                  |
| Joklik and Smith <sup>36</sup>      | (remained with cells)                   |                   | { small amount<br>with bacterium }           |
| Levy <i>et al.</i> <sup>27</sup>    | (most in cells)                         |                   | mainly in fluid                              |
| Kreuger <i>et al.</i> <sup>29</sup> | 20                                      |                   | 80                                           |

\*Phage material stripped from infected bacteria.

Quotes in brackets refer to % isotope remaining with the bacterium, not eluted.

the infected cell. In the next experiments they found that < 1% of the <sup>35</sup>S could be recovered from progeny phage: this moreover was divided among an average of > 30 phage progeny per parent phage. This would not be very convincing if replication was of a non-conservative type but thinking about replication was necessarily vague at this time.

This might have implied that less than 0.03% of the phage protein was necessary for the transfer of genetic information. The most difficult objection would have been if the genetic material had been protein and that this protein had contained little or no sulphur-containing amino-acids. An experimental technique similar to that used by Meselson and Stahl<sup>16</sup> might have resolved this difficulty. The experiment of Meselson and Stahl is itself a blend of Avery's isolation of DNA and of Hershey and Chase's use of isotopic recognition.

## How Hershey and Chase have been quoted

The paper has been reprinted in full in reprint collections<sup>17,18</sup> and, with the omission of pages 39–44 and Tables 1–3 in other collections<sup>19,20</sup>. It is therefore widely available. Most authors quote only the Blender experiment with its results as shown in Table 2. Hayes<sup>21</sup>, Stent<sup>22,23</sup> and Davis *et al.*<sup>11</sup> give details of other experiments in the Hershey and Chase paper. Some authors give accounts of subsequent experiments. Herskowitz<sup>24</sup> gives not the results of the experiment he describes but an idealised interpretation and attributes the account in a footnote "this account follows the work of A. D. Hershey and M. Chase". Diagrams of the experiment are given by Clowes<sup>25</sup> and by Fraser<sup>26</sup>: a diagram in Levy *et al.*<sup>27</sup> is however confusing as it seems that the same phage is labelled with both <sup>32</sup>P and <sup>35</sup>S although the text is clear. Ravin<sup>28</sup> is also confusing, saying that Hershey and Chase "... grew the viruses on bacterial hosts in a medium containing sulphur (<sup>35</sup>S) and phosphorus (<sup>32</sup>P)". A flow diagram of the experiment in Kreuger *et al.*<sup>29</sup> not only obscures the elegance of the original but confuses the reader.

The only reproduction of the figure from the original<sup>1</sup> paper is in Stent<sup>23</sup>—although a figure showing "representative results of the Hershey–Chase 'blender' experiment" is given in Kreuger *et al.*<sup>29</sup>. Stent<sup>22</sup> says that the "Blender experiment ... consists of infecting bacteria with <sup>32</sup>P, <sup>35</sup>S or <sup>14</sup>C labelled phage particles" and details the results as "more than 80% of the viral sulphur and amino-acid carbon ... more than 80% of the viral phosphorus or purine-pyrimidine carbon". The isotope <sup>14</sup>C was used by Watson and Maaløe<sup>10</sup> to label purines in their experiments when they found equal transfer of phosphorus and purines to phage progeny. In the conversations recorded from a conference, Stent<sup>5</sup> quotes the results of the Blender experiment

as "the Hershey–Chase experiment", saying that the experiment was rather weak. This is probably true of that experiment in isolation but it was surely the combination of experiments that made the paper<sup>1</sup> so convincing.

Pollack<sup>30</sup> stated that "with Hershey and Chase's demonstration that bacteriophage left all (that is, experimentally more than 99%) of their protein coat and other superficial 'apparatus' behind on infection of the cell, it became very difficult still to maintain that nucleic acid was not the key substance in the maintenance of genetic continuity". This is a blending of the Blender experiment with the results of the transfer of parental phage radioactive sulphur to progeny phage (1%). Although this is the furthest from accuracy, it nevertheless conveys the general impression of the entire paper more cogently than the quotations of the Blender experiment alone.

A comparison of the figures in Tables 1 and 2 shows that only Stent<sup>23</sup> could have taken his figures from the original (text on page 48). A number of the figures are outside the limits of the results given in the paper from which they are supposed to be taken. The figures of 20% <sup>32</sup>P and 80% <sup>35</sup>S eluted are the most common and are given by Hershey in his summary of the work<sup>9</sup>. It is difficult to believe that so many authors have consulted this paper and not the original. It is more probable that these figures, now part of the myth, stem from an original quotation, now forgotten.

## Effects of idealising experiments

Original experiments are not quoted in textbooks to give authority to the statements and general principles or facts which follow. Rather they are quoted to give a sense of historical perspective to the matter and to relate it to other material. Quoting a name provides a handy label for recall of the matter and perhaps unconsciously a handle for examination questions. Authors may also feel they are sugaring the pill with human interest. Where an experiment is quoted in detail, it is surely to show an example of an elegant economical experiment—a model to strive towards. It should illustrate the clarity of the experimenter's aims and of his reasoning and it should show how far the results meet those required for a definitive answer; it usually shows how a single experiment or a single experiment as the culmination of others, can set scientists on a new path. The very existence of books of selected reprints and their use for teaching<sup>19</sup> shows how important these experiments are considered.

It is therefore of concern that so many textbooks are guilty of trivialising the experiments of Hershey and Chase. By reducing the complementary experiments to only the Blender experiment these textbooks diminish the elegance of the work and destroy the intricacy of the interlocking evidence: each step is in itself inconclusive, but *in toto* they are overwhelmingly convincing. If students accept these textbook accounts they are being taught not science but magic, which can be seen even more clearly in many practical textbooks. A favourite experiment, shown, for example, on a film currently in academic use, consists of precipitating a polymer (presumed to be DNA) and showing that addition of this substance to bacteria increases the number of mutants which can be isolated by selection. It is alleged that this experiment shows that DNA is the specific chemical substance which carries genetic information. As Avery *et al.*<sup>6</sup> showed, the scientific demonstration that DNA is the carrier of genetic information is difficult and laborious (ref. 3 and my unpublished work). We are educating a generation of scientists who may well believe that crucial experiments need no controls and give only rather vague results.

The other aspect of the quotations in Table 2 is evidence of the rapid conversion of scientists to a new dogma. The original evidence is soon forgotten and a gradual process of improvement ensues. The cracks in the edifice are easily overlooked because they are not present in the idealised model. This faith may be necessary so that the majority of scientists can proceed unworried with their work. But it is the young

scientists who can see the cracks and do the next elegant, economical experiment who will found the new dogma.

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# Nitrogenase

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*The protein components of the enzyme system nitrogenase, responsible for the fixation of nitrogen by microbes, have been purified and characterised from several sources. Examination by physicochemical and kinetic methods is revealing an elaborate reaction mechanism involving electron transport from one protein to the other promoted by reaction with Mg-ATP.*

NITROGENASE is the name given to the enzyme system responsible for biological nitrogen fixation, the reduction of dinitrogen ( $N_2$ ) to ammonia<sup>1</sup>. In the past three years, techniques for handling these  $O_2$ -sensitive proteins have improved and sufficient highly purified material has become available for mechanistic studies at the enzyme level. Mössbauer spectroscopy, electron paramagnetic resonance (EPR), rapid reaction kinetics and classical steady state kinetics are converging to advance understanding of the mode of action of the enzyme. The basic action pattern seems to be similar whether the enzyme system is obtained from the strict anaerobe *Clostridium pasteurianum*, the facultative anaerobe *Klebsiella pneumoniae* or the aerobe *Azotobacter vinelandii*. Here we survey the state of the enzymology of nitrogenase at the end of 1973; recent reviews of nitrogen fixation are given in refs 2–5.

## Nitrogenase system

Nitrogenase consists of two proteins, one containing molybdenum, iron and acid-labile sulphur (Mo-Fe protein) and

the other iron and labile sulphur (Fe protein). Both are essential for activity, together with  $Mg^{2+}$  and ATP. ADP is a reaction product; it can inhibit nitrogenase and experimental stratagems are necessary to avoid its accumulation (see ref. 2). High  $Mg^{2+}$  concentrations can inhibit, probably by sequestration of ATP as an inactive  $Mg$ -ATP complex<sup>6</sup>.

In addition to  $N_2$ , which is reduced without formation of detectable intermediates, nitrogenase reduces a variety of triply-bonded nitrogen analogues ( $C_2H_2$ ,  $HCN$ ,  $N_2O$ ,  $CH_3N \equiv C$ ). If no reducible substrate is supplied, hydrogen ions are discharged as  $H_2$ ; this reaction is not completely suppressed by other substrates *in vitro*. Carbon monoxide inhibits all but the hydrogen-evolving function of nitrogenase<sup>2–5</sup>. The reduction of  $C_2H_2$  has been adopted as the now famous 'acetylene test' for nitrogen fixation<sup>7</sup>; the reduction products of the other analogues suggest that substrates bind to a transition metal during enzyme function<sup>2,5</sup>.

Nitrogenase preparations from a number of organisms have been fractionated into two components both of which are required for activity (Table 1). The Mo-Fe proteins from *C. pasteurianum*<sup>8–10</sup>, *K. pneumoniae*<sup>11</sup>, *A. vinelandii*<sup>12,13</sup>, the bacteroid form of *R. japonicum*<sup>14</sup> from soya bean nodules and the Fe proteins of *C. pasteurianum*<sup>10,15</sup> and *K. pneumoniae*<sup>11</sup> have been highly purified.

Nitrogenase components from one organism will often cross react with components from other organisms to give functional enzymes. Table 2 summarises the position at the end of 1973; many of the tests did not use purified nitrogenase components so the table is expressed in only semi-quantitative form.



**Table 1** Organisms from which active nitrogenase has been extracted

| Anaerobic bacteria                              | Comments                                                                                      | References |
|-------------------------------------------------|-----------------------------------------------------------------------------------------------|------------|
| <i>Clostridium pasteurianum</i>                 | Both components characterised                                                                 | Table 3    |
| <i>Desulfovibrio desulfuricans</i>              | Extracts of low activity                                                                      | 55         |
| Facultative anaerobic bacteria                  |                                                                                               |            |
| <i>Klebsiella pneumoniae</i>                    | Both components characterised                                                                 | Table 3    |
| <i>Bacillus polymyxa</i>                        | Two components separated                                                                      | 56         |
| <i>E. coli</i> C-M74                            | Constructed hybrid, Mo-Fe protein immunologically identical with <i>K. pneumoniae</i> protein | 57         |
| Aerobic bacteria                                |                                                                                               |            |
| <i>Azotobacter chroococcum</i>                  | Components separated                                                                          | 58         |
| <i>A. vinelandii</i>                            | Mo-Fe protein characterised                                                                   | Table 3    |
| <i>Mycobacterium flavum</i> 301                 | Two components separated                                                                      | 59         |
| Photosynthetic bacteria                         |                                                                                               |            |
| <i>Chromatium vinosum</i>                       | Mo-Fe protein characterised                                                                   | Table 3    |
| <i>Rhodospirillum rubrum</i>                    | Presumptive Mo-Fe protein only                                                                | 59         |
| Blue-green algae                                |                                                                                               |            |
| <i>Anabaena cylindrica</i>                      | Active extract by sonication                                                                  | 60, 61     |
| <i>Gloecapsa</i>                                | Active extract by sonication                                                                  | 62         |
| <i>Plectonema boryanum</i> 594                  | Active extract by sonication                                                                  | 63         |
| Legume bacteroids                               |                                                                                               |            |
| <i>Glycine max</i> + <i>Rhizobium japonicum</i> | Mo-Fe protein characterised                                                                   | Table 3    |
| <i>Lupinus luteus</i> + <i>R. lupinii</i>       | Active extract by sonication                                                                  | 64         |
| <i>Ornithopus sativus</i>                       | Active extracts by pressure disruption                                                        | 65         |
| <i>Phaseolus aureus</i> } + <i>Rhizobium</i>    | Two components shown                                                                          | 66         |
| <i>Vigna sinensis</i> } spp                     |                                                                                               |            |

Both proteins are irreversibly inactivated by oxygen. The Fe protein is particularly sensitive, therefore purification under an oxygen-free atmosphere is mandatory, and usually  $\text{Na}_2\text{S}_2\text{O}_4$  is included as an  $\text{O}_2$  scavenger in buffer solutions used during purification. Otherwise purification schemes make use of familiar techniques of protein chemistry<sup>5</sup>.

The extreme  $\text{O}_2$  sensitivity of nitrogenase poses a special problem for aerobic  $\text{N}_2$ -fixing organisms, particularly those which are aerobic and/or photosynthetic. Some strains of blue-green algae protect their nitrogenase from oxygen produced during photosynthesis by compartmentation of the enzyme into specialised thick-walled cells termed heterocysts, which lack photosystem II, the  $\text{O}_2$ -producing photosynthetic system. Species of  $\text{N}_2$ -fixing algae exist which do not form heterocysts, but they generally fix  $\text{N}_2$  only at low  $p\text{O}_2$  values or low illumination levels<sup>16</sup>. *Azotobacters* have

an elaborate physiological protection mechanism for their nitrogenase which has been reviewed recently<sup>17</sup>.

They have a very high respiratory rate which is closely coupled to prevailing  $\text{O}_2$  tension in the culture and which provides a dynamic process for lowering the dissolved oxygen concentration to a level at which nitrogenase can function. Extensive  $\text{O}_2$  stress probably causes a 'conformational protection' of  $\text{O}_2$ -sensitive nitrogenase which gives rise to an inactive but  $\text{O}_2$ -tolerant form in the intact organism. Such an  $\text{O}_2$ -tolerant particulate form of nitrogenase is present in crude extracts of *A. vinelandii* and *A. chroococcum*, in which the enzyme proteins are stable in air and become  $\text{O}_2$ -sensitive only during purification or on the addition of ATP or other nucleoside triphosphates<sup>18</sup>. A soluble  $\text{O}_2$ -sensitive nitrogenase can be isolated from azotobacters by osmotic shock<sup>19</sup>;  $\text{O}_2$ -sensitivity of nitrogenase components is apparently influenced by association with membranes, NADH oxidase<sup>20</sup> or ATP.

Ammonia, the product of enzymic nitrogen fixation, does not inhibit nitrogenase activity, but represses synthesis of the nitrogenase system. In *K. pneumoniae* this control occurs at the transcriptional level<sup>21</sup>. Other sources of fixed nitrogen such as  $\text{NO}_3^-$  or urea also inhibit synthesis of nitrogenase if they penetrate the cell. Studies on the regulation of nitrogenase in *A. vinelandii*<sup>22</sup> suggest coordinate control of the level of both nitrogenase components. During repression, activity of the Mo-Fe protein decreases rapidly and at the same rate as its characteristic EPR signal at  $g=3.7$  (below), but immunologically active material persists for much longer.

## Nitrogenase proteins

**The Mo-Fe proteins.** Homogeneous preparations of this protein have been obtained from *C. pasteurianum*, *K. pneumoniae*, soya bean bacteroids, *Chromatium vinosum* and *A. vinelandii*; data are collected in Table 3. It is usually a heteromeric tetramer in free-living bacteria but is homomeric in bacteroids. The protein from *A. vinelandii* has been crystallised<sup>12</sup> but the crystals are too small for X-ray analysis. Crystallisation is an unreliable criterion of purity<sup>13</sup>.

The Fe content ranges from 18–34 Fe atoms per mol, and is approximately equivalent to the acid-labile sulphide content. Reported Mo range from 1–2 atoms per mol. A preparation from *C. pasteurianum* containing 1 Mo atom per mol was separated into a protein with increased activity containing 2 Mo atoms per mol<sup>23</sup>, and an inactive species with 0.5 Mo atoms per mol<sup>24</sup>, the two species were nearly identical in amino acid composition, subunit structure and in immunological reaction. Zumft and Mortenson<sup>24</sup> suggested that other seemingly homogeneous preparations might consist of a mixture of dimolybdo and demolybdoproteins. Nothing is known about the distribution of metals within

**Table 2** Cross-reactions of nitrogenase proteins from various organisms

| Physiological status | Source of Fe protein                 | Av | Ac | Mf | Kp | Bp | Cp | Rr | Acy | Rj | Dd |
|----------------------|--------------------------------------|----|----|----|----|----|----|----|-----|----|----|
| Aerobes              | <i>Azotobacter vinelandii</i> (Av)   | +  | 0  | 0  | +  | —  | —  | 0  | 0   | +  | 0  |
|                      | <i>Azotobacter chroococcum</i> (Ac)  | 0  | +  | +  | +  | +  | 0  | tr | tr  | 0  | 0  |
|                      | <i>Mycobacterium flavum</i> (Mf)     | 0  | +  | +  | +  | +  | 0  | +  | 0   | 0  | 0  |
| Facultative anaerobe | <i>Klebsiella pneumoniae</i> (Kp)    | +  | +  | +  | +  | +  | —  | +  | tr  | 0  | +  |
|                      | <i>Bacillus polymyxa</i> (Bp)        | +  | tr | +  | +  | +  | —  | +  | 0   | +  | +  |
| Anaerobe             | <i>Clostridium pasteurianum</i> (Cp) | —  | 0  | —  | —  | +  | +  | —  | 0   | —  | 0  |
| Photosynthetic       | <i>Rhodospirillum rubrum</i> (Rr)    | 0  | 0  | 0  | 0  | 0  | 0  | +  | 0   | 0  | 0  |
|                      | <i>Anabaena cylindrica</i> (Acy)     | 0  | 0  | 0  | 0  | 0  | 0  | 0  | +   | 0  | 0  |
| Symbiotic            | <i>Rhizobium japonicum</i> (Rj)      | +  | 0  | 0  | 0  | 0  | 0  | 0  | 0   | +  | 0  |

0 = not tested, tr = trace, + = about 50%, — = no activity.

Dd = *Desulfovibrio desulfuricans*. Data from several sources given by Burris<sup>5</sup> and, in addition, refs. 59, 67. Data for Dd from Dr T. H. Blackburn, that for Acy from P. Raynaud (personal communications).

**Table 3** Comparison of the properties of nitrogenase components from various organisms

|                                                  | Mo-Fe protein                     |                                             |                                         |                                                |                                             | Fe protein                        |                                       |
|--------------------------------------------------|-----------------------------------|---------------------------------------------|-----------------------------------------|------------------------------------------------|---------------------------------------------|-----------------------------------|---------------------------------------|
|                                                  | <i>K.pneumoniae</i> <sup>11</sup> | <i>C.pasteurianum</i> <sup>9,23,24,52</sup> | <i>A.vinelandii</i> <sup>12,33,69</sup> | <i>R.japonicum</i> <sup>14</sup><br>bacteroids | <i>Chromatium<br/>vinosum</i> <sup>31</sup> | <i>K.pneumoniae</i> <sup>11</sup> | <i>C.pasteurianum</i> <sup>15,7</sup> |
| Molecular weight                                 | 229,000                           | 220,000                                     | 270,000                                 | 200,000                                        |                                             | 66,700                            | 55,000                                |
| Subunit composition                              | 51,300                            | 50,700                                      | 54,000                                  | 50,000                                         | —                                           | 34,000                            | 27,500                                |
|                                                  | 59,600                            | 59,500                                      | 60,000                                  | one type                                       | two types                                   |                                   |                                       |
| Metal and sulphide<br>content per g atom per mol |                                   |                                             |                                         |                                                |                                             |                                   |                                       |
| Mo                                               | 1                                 | 2                                           | 2                                       | 1.3                                            | 1.3                                         | 0                                 | 0                                     |
| Fe                                               | 18                                | 22–24                                       | 32–36                                   | 29                                             | 17                                          | 4                                 | 4                                     |
| S <sup>2-</sup>                                  | 18                                | 22–24                                       | 28                                      | 26                                             | 14                                          | 3.8                               | 4                                     |
| EPR spectrum                                     | 4.32, 3.63<br>2.009               | 4.27, 3.78<br>2.01                          | 4.3, 3.65<br>2.01                       | —<br>—                                         | 4.3, 3.68<br>2.01                           | 2.053, 1.942<br>1.865             | 2.05, 1.94<br>1.88                    |
| Sensitivity to O <sub>2</sub>                    | +                                 | +                                           | +                                       | +                                              | +                                           | ++                                | +++                                   |
|                                                  | t½ 10 min                         |                                             |                                         | t½ 4.5 min                                     |                                             | t½ 45 s                           |                                       |

the subunits since (i) the metals do not exchange with isotopes and (ii) the treatment with acid or detergent required to dissociate the subunits releases both metals<sup>23</sup>.

All common amino acids are present, (see Table 4) with a high proportion (about 20%) of aspartate and glutamate, which are about twice as abundant as arginine and lysine. The protein from *C. pasteurianum* has a very much lower content of tryptophan than the other proteins and has two C termini, consistent with two different polypeptide subunits. When the compositions are used to estimate compositional relatedness<sup>25</sup>, and to predict the probable degree of sequence homology<sup>26</sup>, the proteins from *A. vinelandii*, *K. pneumoniae* and soya bean bacteroids prove to be more closely related to each other than to that from *C. pasteurianum*. It would be premature to speculate how conservatively the amino acid sequence has been preserved within this group of proteins but the inability of the protein from *C. pasteurianum* to produce an active nitrogenase with complementary proteins from the other organisms (Table 2) might well reflect these differences.

Mo-Fe proteins from *C. pasteurianum*<sup>27,28</sup>, *K. pneumoniae*<sup>29,30</sup>, *Chromatium vinosum*<sup>31</sup> and *A. vinelandii*<sup>12,28</sup> have a unique and characteristic EPR spectrum below 30 K with  $g=2.01$ , 3.7 and 4.3 (Table 3). These resonances are most intense in the presence of Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>, and disappear on oxidation with a dye and also in the functioning nitrogen-fixing system. Palmer *et al.*<sup>27</sup> suggest that they originate from an antiferromagnetically coupled system of iron atoms with an effective spin state of  $S=3/2$ . Molybdenum is, in principle, more likely than Fe to produce this species, but enrichment with <sup>95</sup>Mo (nuclear spin 5/2) produced no hyperfine structure<sup>27,30,31</sup> whereas enrichment with <sup>57</sup>Fe (nuclear spin 1/2) produced some line broadening<sup>30</sup>. Hence Fe atoms are the probable source of the resonances. The precise  $g$  values and line widths of the protein of *K. pneumoniae* are pH dependent, and the substrate analogue C<sub>2</sub>H<sub>2</sub> displaces the signal in favour of the high pH form<sup>30</sup>.

The Mo-Fe protein of *K. pneumoniae* labelled with <sup>57</sup>Fe has been investigated by Mössbauer spectroscopy<sup>32</sup> and three redox states of the undamaged protein can be detected. In the presence of Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>, as normally isolated, the spectrum at 195 or 77 K consists of three overlapping quadrupole pairs of absorptions with relative intensities corresponding to iron sulphur clusters containing 2, 8 and 8 iron atoms. One of the groups of 8 atoms has magnetic properties and has been correlated with the EPR active species. In functioning nitrogenase a more highly reduced form of the Mo-Fe protein appears. Oxidation of the protein gives Mössbauer spectra different from any others, particularly from those observed during catalytic activity; a species formed by oxidation with Lauth's Violet retains full enzyme activity. Extensive O<sub>2</sub> damage results in spectra characteristic of ferric oxide or oxyhydroxide and presum-

ably reflects destruction of the chromophore. A Mössbauer spectrum of the protein from *A. vinelandii*<sup>33</sup> has parameters similar to those of the oxidised protein from *K. pneumoniae*, with possible contributions from O<sub>2</sub>-inactivated protein.

The ultraviolet and visible spectra of the Mo-Fe proteins from all sources<sup>9,11–13,31</sup> are characteristic of iron-sulphur proteins with more than two Fe and S<sup>2-</sup> atoms per molecule. Their solutions are brown and have an absorption maximum near 280 nm. There are no maxima in the visible region, but rather a broad absorption extending from about 350 nm to well into the near infrared. The circular dichroism spectra of the proteins from *K. pneumoniae*<sup>11</sup> and *C. pasteurianum*<sup>34</sup> are very similar, consisting of negative troughs near 210 nm and 220 nm associated with  $\alpha$ -helical structure. They are exceptional among iron-sulphur proteins in that they do not show the optical activity in the visible region found with ferredoxins and xanthine oxidase<sup>35</sup>.

Redox titrations of the proteins from *K. pneumoniae*<sup>36</sup>, and *C. vinosum*<sup>37</sup> indicate a difference of two electrons per mol between the dye-oxidised and Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>-reduced forms but a value of four electrons per mol has been reported for *C. pasteurianum*<sup>38</sup>. The protein of *C. vinosum* has two centres with identical EPR spectra which undergo single electron reductions with midpoint potentials of -60 mV and -260 mV; this differs from *C. pasteurianum* where a four electron reduction at -70 mV is thought to occur<sup>38</sup>.

**The Fe protein.** Only the proteins from *C. pasteurianum*<sup>10,15</sup> and *K. pneumoniae*<sup>11</sup> have been obtained in homogeneous form; their properties are shown in Table 3. The extreme O<sub>2</sub>-sensitivity of these proteins is a major obstacle in purification. The proteins from *A. vinelandii* and *C. pasteurianum*, but not *K. pneumoniae*, are cold labile and lose activity rapidly below 5° C. Fe-proteins are monomeric dimers with similar Fe and S<sup>2-</sup> contents. As with the Mo-Fe proteins, there is a high proportion (about 20%) of acidic amino acid residues which are about twice as abundant as basic residues<sup>11,34</sup>. Although there is a general similarity in composition (Table 4) and a characteristic absence of tryptophan, the compositional relatedness of these proteins is not close.

Electron paramagnetic resonance spectra of the proteins from *C. pasteurianum*<sup>28,29</sup>, *K. pneumoniae*<sup>30</sup> and *A. vinelandii*<sup>28</sup> in the reduced form show a plant ferredoxin-like signal of the  $g=1.94$  type below 40 K, although the line shape indicates a more complex structure than spinach ferredoxin. They integrate to 0.2–0.8 electrons per dimer depending on the source of the protein. The oxidised forms of these proteins have no EPR signal. When Mg<sup>2+</sup> and ATP or ADP are added to the reduced proteins, the symmetry changes from a rhombic to an axial type; other nucleoside triphosphates are inactive. Similar changes in signal symmetry occur if the protein conformation is perturbed by 5 Murea<sup>39</sup>. Titration of the EPR spectrum with ATP showed tight binding of 2 ATP molecules to the protein



**Table 4** Amino acid compositions of nitrogenase components from various organisms

|                       | <i>K.pneumoniae</i> <sup>11</sup> | Mo-Fe protein<br><i>C.pasteurianum</i> <sup>34</sup> | <i>A.vinelandii</i> <sup>33</sup> | <i>R.japonicum</i> <sup>14</sup><br>bacteroids | Fe protein<br><i>K.pneumoniae</i> <sup>11</sup> | <i>C.pasteurianum</i> <sup>68</sup> |
|-----------------------|-----------------------------------|------------------------------------------------------|-----------------------------------|------------------------------------------------|-------------------------------------------------|-------------------------------------|
| Asx                   | 210                               | 192                                                  | 249                               | 182                                            | 60                                              | 24                                  |
| Thr                   | 104                               | 120                                                  | 115                               | 89                                             | 36                                              | 44                                  |
| Ser                   | 110                               | 92                                                   | 134                               | 109                                            | 24                                              | 22                                  |
| Glx                   | 206                               | 196                                                  | 250                               | 179                                            | 84                                              | 68                                  |
| Pro                   | 92                                | 86                                                   | 101                               | 89                                             | 18                                              | 16                                  |
| Gly                   | 152                               | 182                                                  | 206                               | 171                                            | 60                                              | 60                                  |
| Ala                   | 158                               | 140                                                  | 169                               | 162                                            | 60                                              | 36                                  |
| Val                   | 124                               | 148                                                  | 173                               | 126                                            | 42                                              | 32                                  |
| Met                   | 76                                | 60                                                   | 86                                | 39                                             | 36                                              | 16                                  |
| Ile                   | 100                               | 160                                                  | 134                               | 112                                            | 48                                              | 32                                  |
| Leu                   | 182                               | 138                                                  | 190                               | 136                                            | 42                                              | 48                                  |
| Tyr                   | 68                                | 84                                                   | 79                                | 65                                             | 18                                              | 16                                  |
| Phe                   | 96                                | 68                                                   | 102                               | 80                                             | 12                                              | 12                                  |
| His                   | 48                                | 46                                                   | 55                                | 53                                             | 6                                               | 4                                   |
| Lys                   | 102                               | 160                                                  | 177                               | 122                                            | 36                                              | 32                                  |
| Arg                   | 102                               | 62                                                   | 108                               | 94                                             | 24                                              | 24                                  |
| Cys(O <sub>3</sub> H) | 38                                | 40                                                   | 41                                | 23                                             | 18                                              | 12                                  |
| Trp                   | 56                                | 6                                                    | 50                                | 29                                             | 0                                               | 0                                   |
| Total                 | 2,024                             | 1,980                                                | 2,419                             | 1,860                                          | 624                                             | 498                                 |
| % of Glx + Asx        | 20.5                              | 19.5                                                 | 20.6                              | 19.4                                           | 23                                              | 18.4                                |
| % of Lys + Arg        | 10.7                              | 11.2                                                 | 11.8                              | 11.7                                           | 9.6                                             | 11.2                                |
| C termini             | —                                 | Ala, Leu                                             | —                                 | —                                              | —                                               | Leu                                 |

Values given are residues per molecular weight of 229,000 for *K.pneumoniae*; 220,000 for *C.pasteurianum*; 270,000 for *A.vinelandii* and 200,000 for *R.japonicum* bacteroids Mo-Fe protein and 66,700 for *K.pneumoniae* and 55,000 for *C.pasteurianum* Fe proteins.

from *C. pasteurianum*<sup>39</sup>; equilibrium dialysis also showed two binding sites<sup>10</sup>.

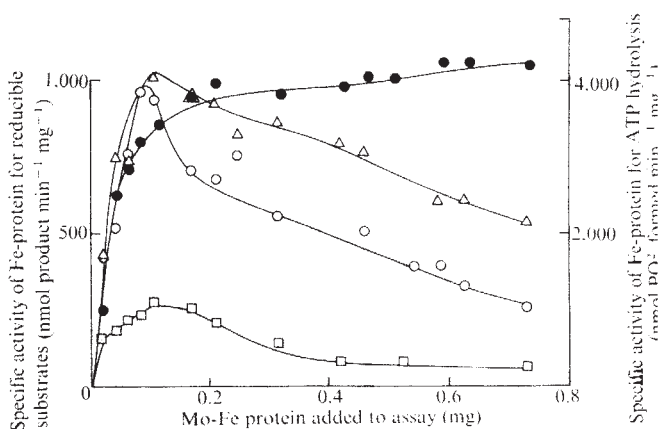
ATP increases the sensitivity of the proteins from *A. chroococcum* or *K. pneumoniae* to O<sub>2</sub>-inactivation<sup>41</sup>. With *K. pneumoniae* Fe protein it produces marked changes in sedimentation behaviour and also enhances the reactivity of sulphhydryl groups with DTNB<sup>42</sup>. The iron of the protein from *C.pasteurianum* reacts faster with the chelating agent  $\alpha,\alpha'$ -dipyridyl in the presence of ATP<sup>43</sup>. A conformational change of the Fe protein produced by ATP binding, presumably alters the symmetry of the iron-sulphur chromophore to a strained configuration, with a more reducing standard potential, from which state electrons are transferred to the Mo-Fe protein. The Mössbauer spectrum of reduced <sup>57</sup>Fe protein from *K. pneumoniae* is consistent with one type of Fe cluster of spin state of S=1/2 with the spin equally distributed over the four iron atoms<sup>32</sup>. Oxygen damage gives rise to a spectrum very similar to that of reduced high potential Fe protein of *Chromatium vinosum* (Hipip), a protein which contains an Fe<sub>4</sub>S<sub>4</sub> cluster<sup>44</sup>. ATP with Mg<sup>2+</sup> causes little change in the Mössbauer spectrum.

The UV/visible spectra of the Fe proteins from *K. pneumoniae*<sup>11</sup> and *C. pasteurianum*<sup>15</sup> are very similar, being featureless in the visible region. Oxidation of the protein from *C. pasteurianum* with O<sub>2</sub> or dyes produces a maximum increase in absorption at 420 nm<sup>38</sup>. Similar changes occur in the N<sub>2</sub>-fixing system when the reductant is exhausted<sup>45</sup>, indicating that it becomes oxidised under these conditions. The proteins from *K. pneumoniae* and *C. pasteurianum* have troughs associated with tertiary structure in the ultraviolet and no visible circular dichroism<sup>11,34</sup>. As noted above for the Mo-Fe proteins, the absence of visible CD is unusual for proteins with iron-sulphur chromophores.

**Electron donors to nitrogenase.** Natural reductants of nitrogenase are ferredoxins and/or flavodoxins. Ferredoxins of N<sub>2</sub>-fixing bacteria have redox potentials below -390 mV but range in iron and sulphur content from 2 to 8 atoms per mol depending on their source<sup>4</sup>. A ferredoxin from one organism will usually show reactivity with nitrogenase from other organisms; flavodoxins have three oxidation states; the hydroquinone-semiquinone couple of *A. chroococcum* flavodoxin is the active electron-donating species to its homologous nitrogenase and also to nitrogenase from *K. pneumoniae*<sup>46</sup>. In anaerobic organisms, pyruvate metabolised by the phosphoroclastic system provides both reduced ferredoxin and ATP; formate or H<sub>2</sub> give only reduced

ferredoxin<sup>4</sup>. Chloroplasts which have been heat-treated to inactivate photosystem II can be used as a reductant for nitrogenase *in vitro* when coupled through a ferredoxin c flavodoxin<sup>47</sup>. Reduced viologen dyes<sup>48</sup> or Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> reduce nitrogenase directly and are widely used *in vitro*.

**Interaction of the nitrogenase proteins.** Though the separated nitrogenase proteins show evidence of interaction with ATP or reducible substrates, neither has yet been shown to catalyse any partial reactions. When increasing amounts of the Mo-Fe protein are added to a fixed amount of Fe protein the nitrogenase activity increases from zero to a plateau if phosphate release from ATP is measured<sup>36,49</sup>, but a region of inhibition is reached for reducible substrates<sup>8,11,49</sup> (Fig. 1) and Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> oxidation<sup>49</sup>. The shape of the inhibition part of the activity titration curve differs according to the source of the enzyme. Experiments with complementary proteins from *K. pneumoniae* and *A. chroococcum* indicate that the Fe protein determines the character of the inhibition<sup>41</sup>. The peak of activity occurs at an equimolar ratio of the two proteins for reduction of N<sub>2</sub> or C<sub>2</sub>H<sub>2</sub> and for H<sub>2</sub> evolution but not for HCN reduction<sup>36</sup>. Activit



**Fig. 1** Nitrogenase activity of *Klebsiella pneumoniae* Fe protein with various concentrations of Mo-Fe protein. Fe protein (42  $\mu$ g) was assayed with various amounts of Mo-Fe protein (for experimental details see ref. 11).  $\bullet$ — $\bullet$ , ATP hydrolysis;  $\square$ — $\square$ , N<sub>2</sub> reduction;  $\circ$ — $\circ$ , C<sub>2</sub>H<sub>2</sub> reduction;  $\triangle$ — $\triangle$ , H<sub>2</sub> evolution. (After Fig. 10 of ref. 11 but including unpublished data for inorganic phosphate release from ATP.)

titration curves have led to the formulation of the stoichiometry of the nitrogenase complex as 1:1 for *K. pneumoniae*<sup>11</sup> and 1:2 (Fe protein in excess) for *C. pasteurianum*<sup>50</sup>. Ultracentrifuge studies<sup>51</sup> of *K. pneumoniae* components also indicate a 1:1 complex because an equimolar mixture, in the absence of  $\text{Na}_2\text{S}_2\text{O}_4$ , sediments as a single peak of 12.4S, compared with the 10.4S of the larger Mo-Fe protein. Additional Fe protein sediments independently at its characteristic S value (4.8).  $\text{Na}_2\text{S}_2\text{O}_4$  prevents formation of the complex species.

The characteristic Mössbauer and EPR spectra of the two proteins have been used to monitor the oxidation state of both proteins during the pre-steady state and during turnover. EPR experiments have been done with components from *K. pneumoniae*<sup>29,30</sup>, *C. pasteurianum*<sup>28,39,50,52,53</sup>, *A. vinelandii*<sup>28</sup> and *Chromatium vinosum*<sup>31</sup>, but Mössbauer experiments have been made only with *K. pneumoniae*<sup>32,34</sup>. The results obtained with nitrogenase from all four sources are gratifyingly consistent, and an early disagreement whether an oxidative<sup>52</sup> or reductive<sup>28-30</sup> change in the Mo-Fe protein occurs has been resolved<sup>38</sup>. Thus the mechanism schematised in Fig. 2 has a sounder experimental basis than those presented in earlier reviews and articles; essentially it implies that the Mg-ATP complex of reduced Fe protein is the electron donor to the EPR active form (intermediate redox state) of the Mo-Fe protein to which the reducible substrate is bound; it is presented for *K. pneumoniae* nitrogenase because most data are available for these proteins but it is probably substantially true of clostridial and other nitrogenases. A summary of the evidence bearing on the steps in Fig. 2 follows.

(1) The electron donor in *K. pneumoniae* has not been identified but it is probably a flavodoxin or ferredoxin as in other nitrogen-fixing bacteria.

(2) The Fe protein accepts electrons from the donor, and binds Mg-ATP to form a species of altered EPR spectrum<sup>30</sup>, tertiary structure<sup>42</sup> and standard potential.

(3) Substrate is bound by the EPR-active form of the Mo-Fe protein at a site which influences the pH-dependence of the EPR spectrum. Since it probably involves a transition metal, it is tempting to assign to Mo the substrate binding role<sup>30</sup>.

(4) In the steady state, the EPR of the Mo-Fe protein decreases by 90%<sup>30</sup> as the fully reduced species appears in the Mössbauer spectrum, indicating that the reduced Mg-ATP-Fe protein transfers electrons to the Mo-Fe protein-substrate species of intermediate redox state<sup>32</sup>. Consistent with this view, the EPR signal of the Mo-Fe protein returns when reductant ( $\text{Na}_2\text{S}_2\text{O}_4$ ) is exhausted<sup>30</sup>.

(5) The Mössbauer spectra of the Fe protein show only slight changes in the functioning enzyme, consistent with some degree of oxidation<sup>32</sup>; the EPR of the reduced form of this protein also persists, though at a lowered intensity<sup>30</sup>.

(6) A 1:1 complex with a half-life longer than the half-life of the redox species of its components in the functional enzyme has been assumed in Fig. 2 on the basis of the activity titration<sup>11,36</sup> and sedimentation data<sup>51</sup> indicated earlier.

The oxidised species of the native Mo-Fe protein which has no EPR but a distinctive Mössbauer spectrum, formed *in vitro* by reaction with the dye Lauth's violet, is rapidly reduced by the reduced Fe protein in the presence of ATP<sup>29</sup>. Such oxidised species probably do not participate in enzyme function *in vivo*.

The scheme in Fig. 2 is naturally imperfect: it supplies only a single function for Mg-ATP and accounts for transfer of only one electron from donor to reducible substrate. It also allows only one reductive site on the enzyme complex, when there may be as many as five<sup>71</sup>. But it provides a basis for future hypotheses, which must incorporate the fact that six electrons are transferred to  $\text{N}_2$ , with the apparent hydrolysis of more than six ATP molecules and without

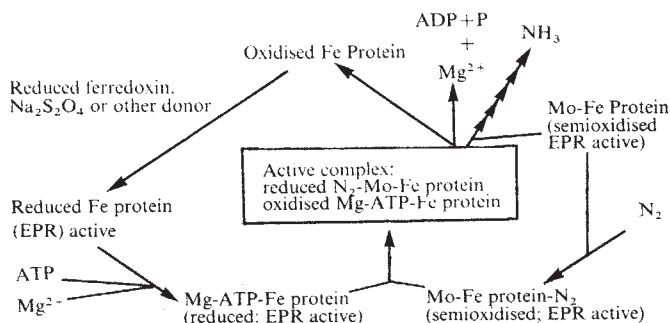


Fig. 2 Schematic representation of events during turnover of nitrogenase. For details see text; (EPR) signifies that the species has an EPR signal; the multiple arrows before  $\text{NH}_3$  signify the six electron transfer steps which occur before an identified reduction product is formed.

identified intermediates. Whether the complex, if real, dissociates and reassociates during catalysis; whether the Fe protein is resupplied with ATP or replaced by new Mg-ATP protein are among the many questions that the scheme poses.

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# Stratospheric sink for chlorofluoromethanes : chlorine atom-catalysed destruction of ozone

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*Chlorofluoromethanes are being added to the environment in steadily increasing amounts. These compounds are chemically inert and may remain in the atmosphere for 40–150 years, and concentrations can be expected to reach 10 to 30 times present levels. Photodissociation of the chlorofluoromethanes in the stratosphere produces significant amounts of chlorine atoms, and leads to the destruction of atmospheric ozone.*

photolytic dissociation to  $\text{CFCl}_2 + \text{Cl}$  and to  $\text{CF}_2\text{Cl} + \text{Cl}$ , respectively, at altitudes of 20–40 km. Each of the reactions creates two odd-electron species—one Cl atom and one free radical. The dissociated chlorofluoromethanes can be traced to their ultimate sinks. An extensive catalytic chain reaction leading to the net destruction of  $\text{O}_3$  and O occurs in the stratosphere:



This has important chemical consequences. Under most conditions in the Earth's atmospheric ozone layer, (2) is the slower of the reactions because there is a much lower concentration of O than of  $\text{O}_3$ . The odd chlorine chain (Cl, ClO) can be compared with the odd nitrogen chain ( $\text{NO}$ ,  $\text{NO}_2$ ) which is believed to be intimately involved in the regulation of the present level of  $\text{O}_3$  in the atmosphere<sup>7–10</sup>. At stratospheric temperatures, ClO reacts with O six times faster than  $\text{NO}_2$  reacts with O (refs 11, 12). Consequently, the Cl–ClO chain can be considerably more efficient than the  $\text{NO}$ – $\text{NO}_2$  chain in the catalytic conversion of  $\text{O}_3 + \text{O} \rightarrow 2\text{O}_2$  per unit time per reacting chain<sup>13</sup>.

## Photolytic sink

Both  $\text{CFCl}_3$  and  $\text{CF}_2\text{Cl}_2$  absorb radiation in the far ultraviolet<sup>14</sup>, and stratospheric photolysis will occur mainly in the 'window' at 1,750–2,200 Å between the more intense absorptions of the Schumann–Runge regions of  $\text{O}_2$  and the Hartley bands of  $\text{O}_3$ .

HALOGENATED aliphatic hydrocarbons have been added to the natural environment in steadily increasing amounts over several decades as a consequence of their growing use, chiefly as aerosol propellants and as refrigerants<sup>1,2</sup>. Two chlorofluoromethanes,  $\text{CF}_2\text{Cl}_2$  and  $\text{CFCl}_3$ , have been detected throughout the troposphere in amounts (about 10 and 6 parts per  $10^{11}$  by volume, respectively) roughly corresponding to the integrated world industrial production to date<sup>3–5,31</sup>. The chemical inertness and high volatility which make these materials suitable for technological use also mean that they remain in the atmosphere for a long time. There are no obvious rapid sinks for their removal, and they may be useful as inert tracers of atmospheric motions<sup>4–6</sup>. We have attempted to calculate the probable sinks and lifetimes for these molecules. The most important sink for atmospheric  $\text{CFCl}_3$  and  $\text{CF}_2\text{Cl}_2$  seems to be stratospheric

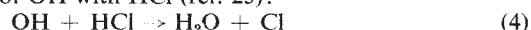
We have extended measurements of absorption coefficients for the chlorofluoromethanes to cover the range 2,000–2,270 Å. Calculations of the rate of photolysis of molecules at a given altitude at these wavelengths is complicated by the intense narrow band structure in the Schumann–Runge region, and the effective rates of vertical diffusion of molecules at these altitudes are also subject to substantial uncertainties. Vertical mixing is frequently modelled through the use of 'eddy' diffusion coefficients<sup>10,15–18</sup>, which are presumably relatively insensitive to the molecular weight of the diffusing species. Calculated using a time independent one-dimensional vertical diffusion model with eddy diffusion coefficients of magnitude  $K \sim (3 \times 10^3) - 10^4 \text{ cm}^2 \text{ s}^{-1}$  at altitudes 20–40 km (refs 10, 15–18), the atmospheric lifetimes of  $\text{CFCl}_3$  and  $\text{CF}_2\text{Cl}_2$  fall into the range of 40–150 yr. The time required for approach toward a steady state is thus measured in decades, and the concentrations of chlorofluoromethanes in the atmosphere can be expected to reach saturation values of 10–30 times the present levels, assuming constant injection at current rates, and no other major sinks. (The atmospheric content is now equivalent to about five years world production at current rates.) Lifetimes in excess of > 10 and > 30 yr can already be estimated from the known industrial production rates and atmospheric concentrations<sup>3,5</sup>, and so the stratospheric photochemical sink will be important even if other sinks are discovered.

Our calculation of photodissociation rates is modelled after those of Kockarts<sup>19</sup> and Brinkmann<sup>20</sup>, and is globally averaged for diurnal and zenith angle effects. The photodissociation rates at an altitude of 30 km are estimated to be  $3 \times 10^{-7} \text{ s}^{-1}$  for  $\text{CFCl}_3$  and  $3 \times 10^{-8} \text{ s}^{-1}$  for  $\text{CF}_2\text{Cl}_2$ , decreasing for each by about a factor of  $10^{-2}$  at 20 km. The appropriate solar ultraviolet intensities at an altitude of 30 km may be uncertain by a factor of 2 or 3 (ref. 21) and we have therefore calculated lifetimes for photodissociation rates differing from the above by factors of 3 or more. The competition between photodissociation and upward diffusion reduces the relative concentration of chlorofluoromethane at higher altitudes and the concentrations should be very low above 50 km. The peak rate of destruction, and formation of Cl atoms, occurs at 25–35 km, in the region of high ozone concentration. The rates of formation of Cl atoms at different altitudes, and the chlorofluoromethane atmospheric lifetimes are sensitive to the assumed eddy diffusion coefficients, as well as to the photodissociation rates.

The major chain processes in the stratosphere involving species with odd numbers of electrons belong to the H (H, OH,  $\text{HO}_2$ ), N ( $\text{NO}$ ,  $\text{NO}_2$ ), and Cl (Cl, ClO) series. ( $\text{ClO}_2$  is rapidly decomposed and its concentration is negligible relative to Cl plus ClO.) These odd-electron chains can only be terminated by interaction with one another, although other reactions can convert one series to another. At most altitudes, the first reaction for converting the Cl–ClO odd-electron chain to an even-electron species containing chlorine is the abstraction of H from  $\text{CH}_4$ , which transfers the odd-electron character to the  $\text{CH}_3$  radical:



At stratospheric temperatures the rate constant for Cl atoms<sup>22</sup>, for (3) is about  $10^{-3}$  times as fast as (1) and the  $\text{O}_3/\text{CH}_4$  concentration ratio can make the rate of (3) less than that of (1) by another factor of 10. The Cl atom chain can be renewed by the reaction of OH with HCl (ref. 23):



Ultraviolet dissociation by absorption in the range 1,750–2,200 Å can also occur at the higher altitudes. The reaction rate of (4) in the stratosphere depends on the concentration of OH, which is known only roughly. In our estimates, termination of the Cl–ClO chain results from downward diffusion of the longer lived species in the chain (ClO, HCl) and eventual removal by tropospheric processes. The rate of termination thus also depends on diffusion processes and estimates will vary with the choice of eddy diffusion coefficients.

Possible terminations involving the Cl series with itself (for example,  $\text{Cl} + \text{ClO} \rightarrow \text{Cl}_2\text{O}$ ) or with one of the others

(for example,  $\text{Cl} + \text{NO} \rightarrow \text{NOCl}$ ) normally lead to molecules with appreciable absorption coefficients at longer wavelengths, which are very rapidly dissociated again by the much more intense solar fluxes available there. Thus, even if a molecule which temporarily terminates two chains is formed, at least one of which involves the Cl series, the terminating molecule is rapidly photolysed and both chains are regenerated again.

Under most stratospheric conditions, the slow reactions in both the Cl–ClO and NO– $\text{NO}_2$  chains occur between O atoms and ClO and  $\text{NO}_2$  molecules. The two chains are interconnected:



The rate of this reaction in the stratosphere is frequently comparable to that of reaction (2). The overall effect is complex and depends on the relative concentrations of  $\text{ClO}_x$ ,  $\text{NO}_x$ ,  $\text{O}_3$ , O and OH. Reaction (1) is so rapid that the ClO/Cl ratio is usually > 10, even when Cl is produced by both reaction (2) and reaction (5), so that the overall rate of reaction (2) is not directly affected by the occurrence of reaction (5). As soon as Cl is produced, however, HCl can form by reactions (1) or (3), resulting in the temporary termination of the Cl atom chain. Whether or not the chain is then restarted depends primarily on the concentration of OH. There are substantial ranges of stratospheric altitudes in which neither reaction (3) nor reaction (5) seriously impedes the chain process of reactions (1) and (2).

The initial photolytic reaction produces one Cl atom from each of the parent molecules, plus a  $\text{CX}_3$  radical (X may be F or Cl). The detailed chemistry of  $\text{CX}_3$  radicals in  $\text{O}_2$  or air is not completely known, but in the laboratory a phosgene-type molecule,  $\text{CX}_2\text{O}$ , is rapidly produced and another X atom—probably Cl (or ClO)—is released from  $\text{CFCl}_2$  or  $\text{CF}_2\text{Cl}$ <sup>24,25</sup>.  $\text{CX}_2\text{O}$  may also photolyse in the atmosphere to give a third and fourth free halogen atom. Thus, each molecule of  $\text{CFCl}_3$  initially photolysed probably leads to between two and three Cl atom chains, and  $\text{CF}_2\text{Cl}_2$  probably produces two Cl atom chains when it is photolysed. Initial calculations suggest that F atom chains will be much shorter than Cl atom chains because the reaction of abstraction from  $\text{CH}_4$  is much faster for F atoms<sup>26</sup>, whereas the reaction between OH and HF is 17 kcalorie  $\text{mol}^{-1}$  endothermic and will not occur in the stratosphere. We have not yet attempted to analyse the subsequent reaction paths of HF.

## Production rates

The 1972 world production rates for  $\text{CFCl}_3$  and  $\text{CF}_2\text{Cl}_2$  are about 0.3 and 0.5 Mton  $\text{yr}^{-1}$  respectively<sup>1,2,5</sup>, and are steadily increasing (by 8.7% per year for total fluorocarbons in the United States from 1961–71) (ref. 1). We have not included any estimates for other chlorinated aliphatic hydrocarbons also found in the atmosphere, such as  $\text{CCl}_4$  (refs 3 and 4),  $\text{CHCl}_3$ ,  $\text{C}_2\text{Cl}_4$  and  $\text{C}_2\text{HCl}_3$  for which there is no evidence for long residence times in the atmosphere<sup>27</sup>. If the stratospheric photolytic sink is the only major sink for  $\text{CFCl}_3$  and  $\text{CF}_2\text{Cl}_2$ , then the 1972 production rates correspond at steady state to globally averaged destruction rates of about  $0.8 \times 10^7$  and  $1.5 \times 10^7$  molecules  $\text{cm}^{-2} \text{ s}^{-1}$  and formation rates of Cl atoms of about  $2 \times 10^7$  and  $3 \times 10^7$  atoms  $\text{cm}^{-2} \text{ s}^{-1}$ , respectively. The total rate of production of  $5 \times 10^7$  Cl atoms  $\text{cm}^{-2} \text{ s}^{-1}$  from the two processes is of the order of the estimated natural flux of NO molecules ( $2.5 - 15 \times 10^7$  NO molecules  $\text{cm}^{-2} \text{ s}^{-1}$ ) involved in the natural ozone cycle<sup>9–12</sup>, and of the  $5 \times 10^7$  NO molecules  $\text{cm}^{-2} \text{ s}^{-1}$  whose introduction around 25 km from stratospheric aviation is estimated would cause a 6% reduction in the total  $\text{O}_3$  column<sup>10</sup>.

Photolysis of these chlorofluoromethanes does not occur in the troposphere because the molecules are transparent to wavelengths longer than 2,900 Å. In fact the measured absorption coefficients for  $\text{CFCl}_3$  and  $\text{CF}_2\text{Cl}_2$  are falling rapidly at wavelengths longer than 2,000 Å (ref. 14). The reaction between OH and  $\text{CH}_4$  is believed to be important in the troposphere<sup>17,28</sup>, but the corresponding Cl atom abstraction reaction (for example,  $\text{OH} + \text{CFCl}_3 \rightarrow \text{HOCl} + \text{CFCl}_2$ ) is highly endothermic and is negligible under all atmospheric conditions.



Neither  $\text{CFCl}_3$  nor  $\text{CF}_2\text{Cl}_2$  is very soluble in water, and they are not removed by rainout in the troposphere. Details of biological interactions of these molecules in the environment are very scarce because they do not occur naturally (except possibly in minute quantities from volcanic eruptions)<sup>29</sup>, but rapid biological removal seems unlikely. The relative insolubility in water together with their chemical stability (especially toward hydrolysis)<sup>30</sup> indicates that these molecules will not be rapidly removed by dissolution in the ocean, and the few measurements made so far indicate equilibrium between the ocean surface and air, and therefore a major oceanic sink cannot be inferred<sup>3</sup>.

It seems quite clear that the atmosphere has only a finite capacity for absorbing Cl atoms produced in the stratosphere, and that important consequences may result. This capacity is probably not sufficient in steady state even for the present rate of introduction of chlorofluoromethanes. More accurate estimates of this absorptive capacity need to be made in the immediate future in order to ascertain the levels of possible onset of environmental problems.

As with most  $\text{NO}_x$  calculations, our calculations have been based entirely on reactions in the gas phase, and essentially nothing is known of possible heterogeneous reactions of Cl atoms with particulate matter in the stratosphere. One important corollary of these calculations is that the full impact of the photodissociation of  $\text{CF}_2\text{Cl}_2$  and  $\text{CFCl}_3$  is not immediately felt after their introduction at ground level because of the delay required for upward diffusion up to and above 25 km. If any Cl atom effect on atmospheric  $\text{O}_3$  concentration were to be observed from this source, the effect could be expected to intensify for some time thereafter. A lengthy period (of the order of calculated atmospheric lifetimes) may thus be required for natural moderation, even if the amount of Cl introduced into the stratosphere is reduced in the future.

This research has been supported by the US Atomic Energy Commission. We acknowledge a helpful discussion with Professor H. S. Johnston.

Received January 21; revised March 21, 1974.

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# LETTERS TO NATURE

## PHYSICAL SCIENCES

### X-ray spectrum of NP0532

THERE is a change in the X-ray spectral index of the Crab pulsar NP0532 near 10 keV (refs 1 and 2). We here examine this in a more quantitative fashion than before, using most of the available published data, together with some unpublished data (Table 1). The pulsed fraction is the ratio of the pulsed radiation to total radiation; data published as the ratio of the pulsed to unpulsed components have been duly corrected. The unpulsed reference level has been determined according to Ducros *et al.*<sup>3</sup> and where other workers have used a significantly different convention, these data have been corrected. We believe that all data (31 points), can be validly compared. Other results, not considered here<sup>2,4,5</sup>, are generally in agreement with the data we have used. The use of the pulsed fraction as the basic spectral quantity allows the low energy spectrum to be treated independently of interstellar absorption because it is the same for both the pulsed and unpulsed components.

A multiparameter, nonlinear, weighted routine was used to

fit least squares (see refs 6 and 7). This program yields values of the unknown parameters of any generalised mathematical model which is applied to input data, together with relevant statistics, including the approximate 95% confidence interval for each parameter and the reduced  $\chi^2$ , the value of  $\chi^2$  per degree of freedom) for the successful fit.

Any change in spectral slope will probably be gradual, perhaps spanning a decade or more. The precision of the available data is, however, such that a sharp break can be assumed for the source model, at least initially. This is also analytically simple. The assumption of identical spectral indices above the break energy is justified in a similar way (see also ref. 8). The pulsed fraction  $F$  as a function of energy  $E$  (keV) is thus given by

$$F(E) = \begin{cases} A, & E \geq E_B \\ B \exp(-\beta \lambda^2 E \gamma - \alpha), & E < E_B \end{cases}$$

where  $A$  is the fraction (assumed constant) above the break

**Table 1** Summary of the data used\*

| Source                   | Energy range (keV) | No. of data points |
|--------------------------|--------------------|--------------------|
| a, Saclay (unpublished)  | 0.7–10             | 11                 |
| b, NRL <sup>1</sup>      | 0.6–9              | 6                  |
| c, Caltech <sup>12</sup> | 0.7–2.5            | 2                  |
| d, MIT <sup>13</sup>     | 1.5–10             | 1                  |
| e, GSFC <sup>14</sup>    | 2–10               | 1                  |
| f, Saclay <sup>3</sup>   | 2.2–30             | 4                  |
| g, MIT <sup>15</sup>     | 25–100             | 1                  |
| h, Rice <sup>8</sup>     | 42–100             | 3                  |
| i, Saclay <sup>16</sup>  | 15–150             | 2                  |

\*Values are derived for the size and location of the break, assuming identical spectral indices for the pulsar and the nebula above the break energy and taking into account the effect of interstellar dust scattering on the pulsed radiation below approximately 2 keV. a, Unpublished data (C. Jehanno, C. Cheron, J. Leloup, R. Rothenflug, and R. Thomas).

energy  $E_B$ ,  $\alpha$  and  $\gamma$  are the spectral indices of the pulsar and total emission energy respectively,  $\beta(\text{\AA}^{-2})$  is the interstellar grain scattering coefficient,  $\lambda$  is the photon wavelength ( $\text{\AA}$ ), and  $B = A \exp(\beta \lambda_B^2) E_B \alpha^{-\gamma}$  where  $\lambda_B = hc/E_B$ . The effect of scattering is felt only below about 2 keV, and so  $\beta$  is left a fixed parameter. An X-ray measurement of this quantity, and thus of the amount of dust in the line of sight should, however, become possible with further improvement in the precision of 0.5 and 2 keV data. Consequently, two fixed values of  $\beta$  were tried:  $\beta = 0$  (no scattering) and  $\beta = 2 \times 10^{-3} \text{\AA}^{-2}$  (as expected in the direction of the Crab)<sup>9</sup>. The quantities  $\alpha$ ,  $A$  and  $E_B$  were left as free parameters, with  $\gamma = 1.0$ . The computational results are summarised in Table 2 and the best fit is drawn through the data of Fig. 1.

The choice of  $\beta$  makes little difference to the quality of the fit, but using  $\beta = 2 \times 10^{-3} \text{\AA}^{-2}$  makes the spectral index and

**Table 2** Computational results for parameters used

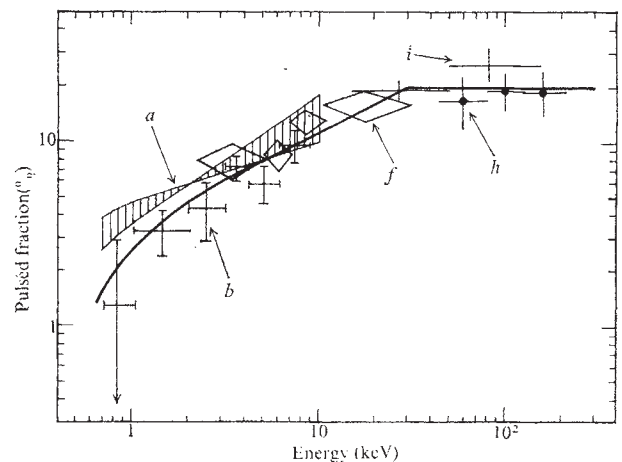
| $\beta(\text{\AA}^{-2})$ | $\chi_r^2$ | $\alpha$        | $E_B(\text{keV})$ | $A(\%)$    |
|--------------------------|------------|-----------------|-------------------|------------|
| 0                        | 0.75       | $0.35 \pm 0.15$ | $24 \pm 13$       | $19 \pm 4$ |
| $2 \times 10^{-3}$       | 0.72       | $0.44 \pm 0.13$ | $30 \pm 17$       | $20 \pm 4$ |

the break energy slightly greater. The large error in  $E_B$ , together with the acceptable  $\chi_r^2$  value, reflects the lack of precision of the data. A sharp break at  $30 \pm 17$  keV satisfies the data well, although statistically improved data will probably modify this conclusion in the future.

The value  $\alpha = 0.44 \pm 0.13$  is derived relative to a total emission index of  $\gamma = 1.00$ . Taking the error in  $\gamma$  to be  $\pm 0.1$  will yield a final result of  $\alpha = 0.4 \pm 0.2$ . This result is not inconsistent with the suggestion<sup>10</sup> that the pulsar X-ray index may be identical to the Nebula radio index of 0.26, indicating a common parent electron population in regions with different magnetic fields. A spectral index of between 0.4 and 0.5, extrapolated toward the optical region, agrees, however, much better with the known pulsar optical spectrum than does an index of 0.26 or 0.2 (ref. 1).

The present fit shows that there is no necessity to postulate a further sharp change of spectral index in NP0532 between 1.5 and 3 keV as suggested by Rappaport *et al.*<sup>4</sup>

The idea that the decrease in the pulsed fraction below the break is attributable entirely to interstellar scattering (ref. 11) was tested. The resulting fit was unacceptable ( $\chi_r^2 = 2.7$ ) and the derived value of  $\beta = (1.6 \pm 0.9) \times 10^{-2} \text{\AA}^{-2}$  was an order of magnitude higher than optical measurements imply (ref. 9), regardless of the grain model assumed. This model can therefore be rejected (see also refs 3 and 9).



**Fig. 1.** The X-ray pulsed fraction of NP0532, showing the least squares fit. A selection of the data listed in Table 1 is displayed. Notation as in Table 1. Solid curve, line of best fit.

Variations of the total emission spectral index of the Nebula with energy<sup>2</sup>, are relevant. Our assumption that the total emission spectral index is given by  $\gamma = 1.0 \pm 0.1$  remains valid for energies up to 60 keV (refs. 1 and 2), a fact which lends confidence to the derived value of  $\alpha = 0.4 \pm 0.2$  for the pulsar below about 30 keV. The increase in  $\gamma$  to  $1.46 \pm 0.11$  above 60 keV brings into question the assumption that  $\alpha = \gamma$  for  $E > E_B$ , which would imply a second pulsar break at 60 keV, for which there is no evidence. In our opinion, however, the use of the analytically simple model presented here is justified at this time by the relatively poor statistics of the higher energy pulsar data.

R.M.T. acknowledges the hospitality of Professor J. Labeyrie of Saclay and the support of the Rothmans University Endowment Fund at the University of Tasmania. We thank Dr T. Palmieri for help.

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Received March 4; revised April 2, 1974.

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## Charge composition of cosmic rays between 4 and 100 GV

SEVERAL groups have reported the energy dependence of the charge composition of cosmic-ray nuclei with  $Z \geq 3$  (refs 1-5). The results suggest that the ratio of the numbers of nuclei in groups L and M (defined in Table 1) decreases as the rigidity increases. At 100 GV the ratio is about 25% lower than the value below 10 GV. There is, however, no general agreement about the variation with rigidity of the ratio of VH nuclei to M nuclei; a variable ratio would be evidence for the existence of more than one basic acceleration mechanism, because both charge groups are thought to consist mainly of particles coming directly from the sources. The largest variation in the ratio of VH to M nuclei is reported by Ormes and Balasubrahmanyam<sup>1</sup>. We report here the measurement of the ratios of L nuclei to M

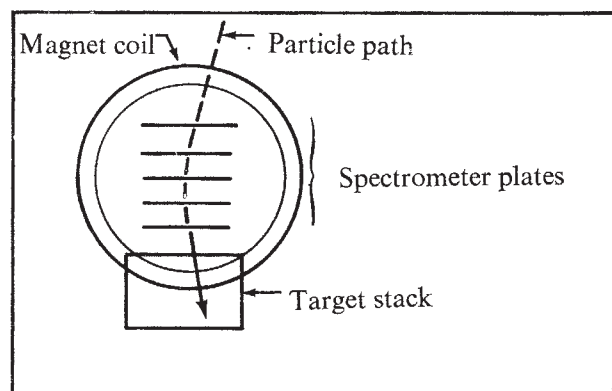


Fig. 1 Schematic representation of the detector system indicating the location of the five spectrometer plates and the target emulsion stack.

nuclei and of VH nuclei to M nuclei using a magnetic spectrometer. Our results are based on two balloon flights, the first from Palestine, Texas, on August 23, 1969, and the second on November 13, 1970, from Parana, Argentina. The combined exposure factor for these flights was  $273 \text{ cm}^2 \text{ sr s}$  at an average altitude of  $3 \text{ g cm}^{-2}$ .

The apparatus<sup>6</sup> consists of an emulsion block and five double-coated glass plates arranged in the field of a superconducting magnet (Fig. 1). Events were found by area scanning the target block and rigidities were measured by tracing particles through the emulsion-coated glass plates. The scanning efficiency was  $> 95\%$ . The process of rigidity measurement has a resolution of

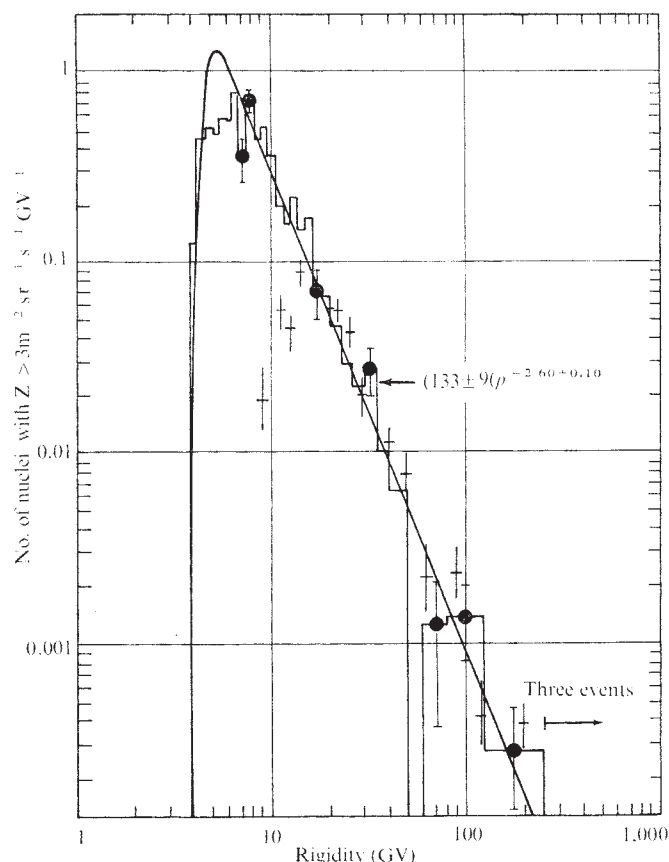


Fig. 2 Rigidity spectrum. The histogram contains data from the Palestine flight; the data bars are from the Parana flight. Both curves are uncorrected for geomagnetic transmission. ●, Palestine flight; + Parana flight.

12% at 5 GV and 35% at 100 GV (ref.<sup>6</sup>); it is very straightforward (using a 14-constraint least squares fit to each track) and does not rely on assumptions concerning observed spectrum. Alignment and resolution information are obtained by turning off the magnet in flight to calibrate the system with tracks which are straight except for Coulomb scattering. A computer-controlled microscope with a measuring accuracy of  $\pm 2.5 \mu\text{m}$ , was used to measure the trajectories. A total of 791 tracks of nuclei with  $Z \geq 3$  were located during the area scan. This paper is based on the 625 events which have so far been measured completely. In

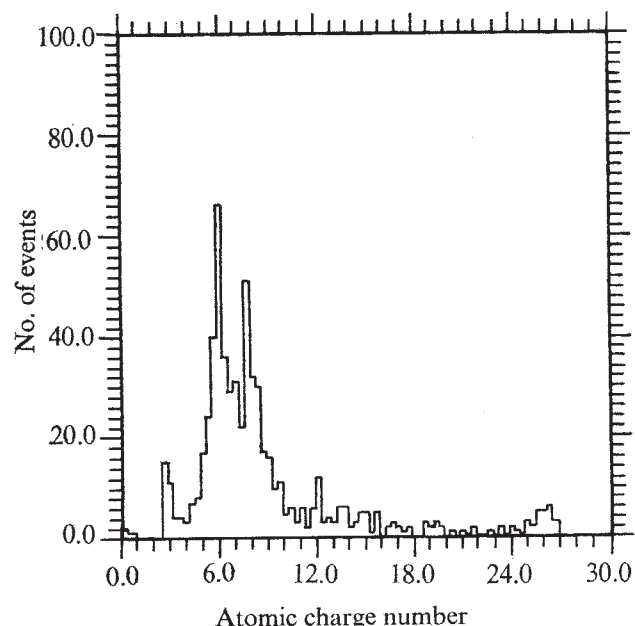


Fig. 3 Charge composition of the combined flight data.

Table 1 Number of nuclei as a function of charge group and rigidity

| Charge group                   | Rigidity interval (GV) |      |       |       |       |      |
|--------------------------------|------------------------|------|-------|-------|-------|------|
|                                | 4-7                    | 7-10 | 10-15 | 15-25 | 25-50 | > 50 |
| L ( $3 \leq Z \leq 5$ )        | 18                     | 21   | 18    | 21    | 7     | 8    |
| M ( $6 \leq Z \leq 8$ )        | 74                     | 64   | 81    | 78    | 49    | 35   |
| LH + MH ( $9 \leq Z \leq 19$ ) | 22                     | 29   | 18    | 26    | 18    | 5    |
| VH ( $20 \leq Z \leq 27$ )     | 7                      | 7    | 7     | 5     | 1     | 3    |

Total no. of events = 625. LH,  $9 < Z < 14$ ; MH,  $15 < Z < 19$ .

Fig. 2 we present the rigidity spectra of nuclei with  $Z \geq 3$  gathered during the two flights. There is close agreement between the energy spectra of Texas and Argentina flights at rigidities above 12 GV, where solar modulation should be negligible. The absolute flux value agrees with that of Anand *et al.*<sup>7</sup>. A maximum likelihood analysis gives a spectral index of  $-2.6 \pm 0.1$ , which is consistent with other observations in this region.

Charge measurement was carried out using a television microscope to measure track area. The charge distribution obtained with the microscope photometer is shown in Fig. 3. The resolution is 0.4 charge units at carbon and  $\sim 1.0$  charge unit at iron. Clearly, there is some contamination of L nuclei from the neighbouring carbon peak. This contamination should be independent of rigidity.

Figures 4 and 5 show the charge composition as a function of rigidity. The data are uncorrected for the  $3 \text{ g cm}^{-2}$  of air,  $3 \text{ g cm}^{-2}$  of emulsion and  $1 \text{ g cm}^{-2}$  of glass above the scan line.

Measurement of spectral indices for the individual charge groups is difficult at rigidities below 12 GV because of very

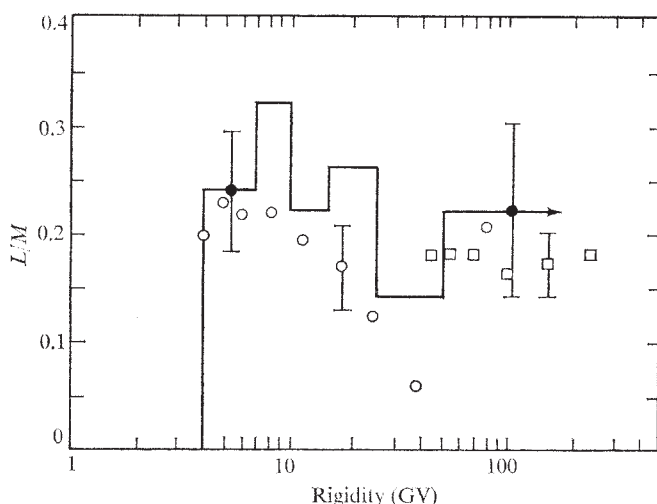


Fig. 4 Ratio of L nuclei to M nuclei as a function of rigidity.  $\circ$ , Smith *et al.*<sup>4</sup>;  $\square$ , Juliussen *et al.*<sup>2</sup>.

strong solar modulation during the Texas flight and a geomagnetic cutoff at 12 GV during the second flight. Instead of measuring separate spectral indices we have measured the dependence on rigidity of the ratios of the charge groups. This rests on the implicit assumption that effects arising from solar modulation and geomagnetic cutoff do not depend on charge in the range 4–12 GV. The analysis of the dependence on rigidity of the charge ratios was carried out by using the M nuclei at a given rigidity to predict the numbers of VH or L nuclei. For a given charge group (VH, for example) a charge ratio relative to the M group  $R = ap^{-\Delta K}$  is assumed. The constant  $a$  is chosen so that the number of expected VH (or L) nuclei equals the number of observed VH (or L) nuclei. The parameter  $\Delta K$  is the difference in spectral indices ( $\Delta K > 0$  means VH is flatter than M). In each rigidity interval

$$\langle VH \rangle_{\text{expected}} = R(\bar{p}, \Delta K) (M)_{\text{observed}} \quad (1)$$

Using the values of  $\langle VH \rangle_{\text{exp}}$  and  $(VH)_{\text{obs}}$  a pseudo-likelihood function is evaluated for a given value of  $\Delta K$  by multiplying the Poisson probabilities of all the rigidity intervals.

$$L(\Delta K) = \prod_{\text{rigidity intervals}} P(\langle VH \rangle_{\text{exp}}, (VH)_{\text{obs}}) \quad (2)$$

where  $P$  is the Poisson probability of observing  $(VH)_{\text{obs}}$  when  $VH_{\text{exp}}$  is the expected number of counts.

The effects of mixing of data from adjacent rigidity intervals

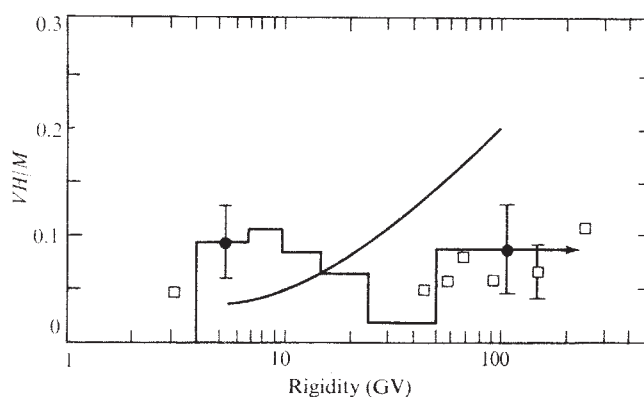


Fig. 5 Ratio of VH nuclei to M nuclei as a function of rigidity.  $\square$ , Juliussen *et al.*<sup>2</sup>; curve, best fit for  $VH/M$  after Ormes and Balasubrahmanyam<sup>1</sup>.

are small for the magnetic spectrometer. The uncertainty in measured rigidity is easily determined by least-squares error propagation. The largest correction would be to the highest rigidity bin in the ratio of VH nuclei to M nuclei. The ratio should be lowered by  $\sim 0.01$  which is negligible. Although such corrections may be significant for other less accurate energy-measuring devices they can be neglected in this analysis.

The likelihood analysis yields a spectral index difference  $\Delta K = -0.2 \pm 0.2$  for the  $VH/M$  ratios. Our result is in strong conflict with that of Ormes and Balasubrahmanyam<sup>1</sup>,  $+0.57 \pm 0.14$ , which has a likelihood of 0.1% relative to our most probable result. This result is not inconsistent with the observations of Juliussen *et al.*<sup>2</sup>, Webber *et al.*<sup>3</sup> and Atallah *et al.*<sup>4</sup> as can be seen in Fig. 5. Our experiment itself, however, reveals no evidence of a difference between the VH spectrum and the M spectrum. The L/M analysis gives a spectral index difference  $\Delta K = +0.12 \pm 0.4$ , consistent with the observations of all other observers but also consistent with zero. It is worth noting again that our L sample is contaminated with 20–25% of carbon nuclei. The  $(LH + MH)/M$  ratio gave a spectral index difference of  $-0.14^{+0.1}_{-0.16}$ .

Since the area scan is 95% efficient, the identification of particles is independent of energy and the rigidity measurement relies only on the solution of the Lorentz-force equation, this experiment can be expected to be exceptionally free of energy-charge biases. We find no evidence that the VH spectrum is steeper than the M spectrum. The difference in spectral indices is  $-0.2 \pm 0.2$  (minus meaning VH is steeper than M). Observations of the L and  $(LH + MH)$  fluxes relative to the M flux is consistent with the observations of the Berkeley and Chicago groups.

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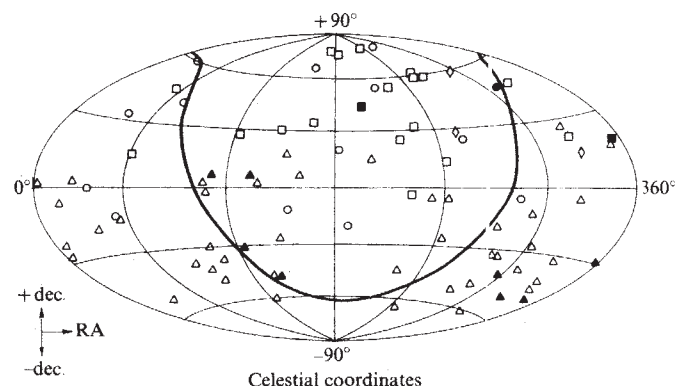
## Isotropy of the arrival directions of ultra high energy cosmic rays

THERE has been little success in the search for anisotropies in the arrival directions of ultra high energy cosmic rays. Data on 50 events of very high energy, having an arrival direction which is 'not obviously isotropic', have however, been collected by the Sydney extensive air shower group<sup>1</sup>. The data were recorded over a period of 5 yr and, as the world total of such events is likely to increase only rather slowly, this seems to be the appropriate time to combine these data with comparative data from the

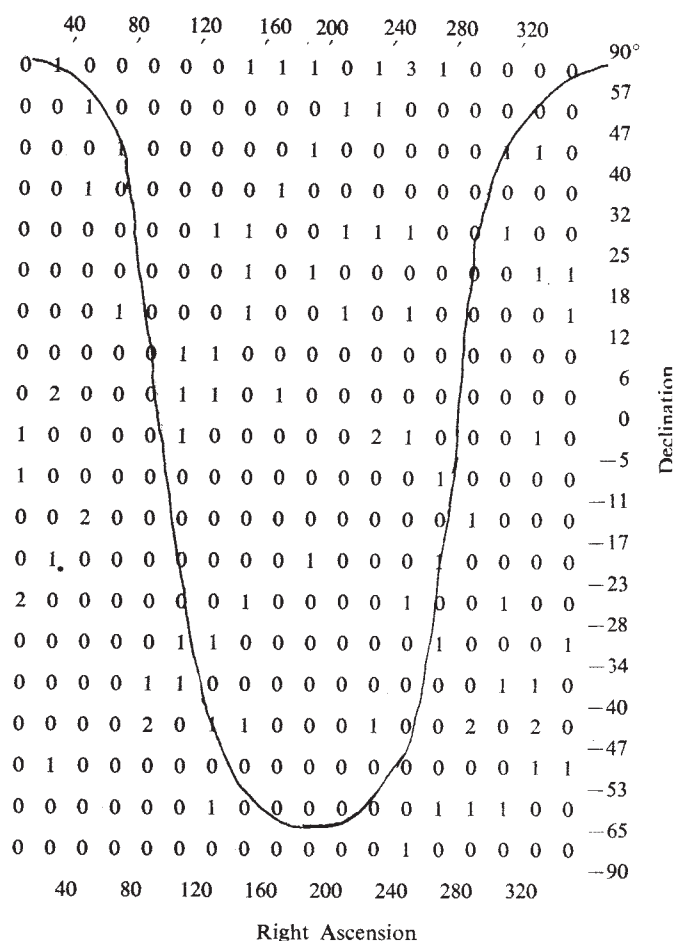
**Table 1** Comparison of observed and predicted number of equi-event areas

| No. of events per square | Observed no. of squares assuming Poissonian distribution | Expected no. of squares assuming Poissonian distribution |
|--------------------------|----------------------------------------------------------|----------------------------------------------------------|
| 0                        | 285                                                      | 284.8                                                    |
| 1                        | 67                                                       | 66.5                                                     |
| 2                        | 7                                                        | 7.76                                                     |
| 3                        | 1                                                        | 0.6                                                      |
| 4                        | 0                                                        | 0.04                                                     |

Northern Hemisphere arrays operated at Cornell (United States), Haverah Park (United Kingdom) and Volcano Ranch (United States). The arrival directions of 87 showers with primary energies above a few times  $10^{19}$  eV are discussed here. It is shown that there are no large deviations from an isotropic distribution and it is concluded that the ultra high energy cosmic rays are most likely to come predominantly from extra-Galactic sources.



**Fig. 1** Aitoff equal area projection of 87 events<sup>1,3-6</sup> in celestial coordinates. Solid line, Galactic plane; triangles, Sydney observations (50); circles, Volcano Ranch observations (14); squares, Haverah Park observations (20); diamonds, Cornell observations (3). Events which may have primary energies above  $10^{20}$  eV have the appropriate symbol filled. Energy assignment has been made using a model of shower development described by Hillas (see ref. 4 for details).



**Fig. 2** Equi-event plot in celestial coordinates of 84 events<sup>1,4-6</sup>. Areas of  $20^\circ \text{ RA} \times (\delta_i - \delta_j)^\circ \text{ dec.}$  are expected to contain equal numbers. The values of  $\delta_i, \delta_j$  are listed at the right hand side of the figure and are deduced from the declination distributions<sup>1,5,6</sup> by assuming that the exposure time of each experiment is proportional to the number of events recorded. The solid line represents the Galactic plane.

The events discussed by the Sydney group are all of higher primary energy than a threshold level which is claimed to be  $1.5 \times 10^{19}$  eV. The assignment of energy is subject to the energy conversion which is used to relate the measured number of muons in their showers to primary energy. Less ambiguity in the threshold of selection of data from different arrays is therefore introduced if only those events are included which were recorded at an intensity less than a certain value. Using the energy spectrum given by the Sydney group<sup>2</sup> their threshold intensity is found to be  $4.62 \times 10^{-15} \text{ m}^{-2} \text{ s}^{-1} \text{ sr}^{-1}$ . Events from the Cornell, Haverah Park and Volcano Ranch data banks were therefore selected only if they were recorded at intensities less than this value. Of the additional 37 events recorded by the Northern Hemisphere experiments, three were recorded at Cornell (refs 3, 4 and J. Delvaile, personal communication), 20 were recorded at Haverah Park<sup>5,6</sup> and 14 at Volcano Ranch. The directional uncertainty varies slightly between experiments but is believed to be better than  $3 \times 10^{-3} \text{ sr}$  for all 87 events.

All 87 events are shown on an Aitoff equal-area projection in celestial coordinates (Fig. 1). The Galactic plane is also indicated. It is clear that there is no evidence of significant clustering of events. In particular the region close to the Galactic plane shows no indication of the excess which could be

expected if the sources of high energy cosmic rays were objects (for example, pulsars) which are strongly clustered on the Galactic plane. Statistical support can be given to the conclusion that the data are consistent with isotropy if a rectangular right ascension (RA)—declination (dec.) plot is divided into areas within which the number of observed events would be expected to be equal. For the Volcano Ranch, Haverah Park and Sydney arrays sufficient data have been published<sup>1,6,7</sup> at low energies ( $10^{17}$ – $10^{18}$  eV), where the evidence of isotropy is very strong, to enable such an 'equi-event' diagram to be constructed, and Fig. 2 shows 360 'equi-event' areas. The mean number of events expected per area is 0.234 and the number of areas with 0, 1, 2, 3, . . . events is compared with the predicted number in Table 1 assuming that the arrival directions are randomly distributed. The data are clearly consistent with isotropy of arrival directions and contain no evidence for the existence of point sources of ultra high energy cosmic rays.

It seems likely that primary cosmic rays in the energy region in question ( $E_p \geq 2 \times 10^{19}$  eV) have atomic masses  $1 \leq A \leq 56$ , but no experimental data are available to enable this range to be narrowed. Karakula *et al.*<sup>8</sup> have estimated the extended anisotropies which would be likely to arise in various Galactic magnetic field models if the cosmic ray sources were concentrated in the disk or the spiral arms of the Galaxy, and if the primary particles were protons. Comparing the data of Fig. 1 with the distributions predicted by these authors for protons of  $10^{19}$  eV and several reasonable Galactic field models, we conclude that if the primaries are protons then ultra high energy cosmic rays are predominantly of extra-Galactic origin.

If the primary particles at these energies are iron nuclei then the situation is not quite so clear cut. For a model in which the Galactic field is longitudinal but has opposite orientations above and below the Galactic plane, a strong peak is predicted<sup>9</sup> in the direction of the inward spiral arm (RA =  $308^\circ$ , dec. =  $35^\circ$ ) for iron nuclei of momentum  $> 2.10^{19}$  eV/c, which is clearly in contradiction with the observations. For a simple longitudinal field, which seems to be favoured at the present time<sup>10</sup>, some extra-Galactic contribution (probably greater than 30%) seems to be necessary. We therefore conclude that even if the primary particles are iron nuclei a significant fraction of them ( $> 30\%$ ) come from extra-Galactic sources.

The data discussed in this paper include about 11 events with a primary energy of more than  $10^{20}$  eV (Fig. 1) but it is important to note that the absence of a cutoff in the energy spectrum<sup>1,5,6</sup> is not evidence in favour of a Galactic origin for them. Stecker<sup>11</sup> has pointed out that the absence of a cutoff implies only that the lifetimes of these cosmic rays are significantly less than the age of the Universe, and consequently that sources in the local 'super-cluster' of galaxies must be considered possible.

Support by the Science Research Council and the National Science Foundation is acknowledged. A. A. W. thanks Professor J. G. Wilson and Dr R. J. O. Reid for discussion.

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Received February 14; revised March 12, 1974.

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## NO and O<sub>3</sub> measurements in the lower stratosphere from a U-2 aircraft

AIRBORNE nitric oxide and ozone detectors have been carried on aircraft flights over the west coast of the United States to obtain *in situ* measurements of nitric oxide and ozone at altitudes up to 21 km. Results from two of these flights—December 6, 1973 from 1100 to 1430 local time (1900–2230 UT) and December 18, 1973 from 1030 to 1430 local time (1830–2230 UT)—are presented here. The experimental platform was one of two U-2 aircraft used in the NASA Earth Resources Aircraft Project at Ames Research Center, Moffett Field, California.

The nitric oxide instrument is a chemiluminescent detector based on the luminescent reaction of nitric oxide with excess ozone in a flow reactor. The photon count rate signals from the detector are proportional to the atmospheric nitric oxide mixing ratio and to the STP flow rate of the atmospheric sample. Two different calibrator flow rates from an on-board NO/N<sub>2</sub> calibrator bottle provide periodic instrument sensitivity checks. The zero level was periodically measured by mixing the excess ozone with the atmospheric sample upstream of the reactor. Signals were derived from two linear count rate circuits, each having a time constant of about 1 s. An automatic cycle timer sequenced the instrument through the measure, calibrate and zero level modes for 3 min each. The sensitivity of the nitric oxide detector for the measurements reported here is 0.025 parts in 10<sup>9</sup> by volume.

Ozone concentration measurements are based on the absorption of the Hg line at 2,537 Å in the Hartley continuum. The unattenuated mercury lamp intensity at 2,537 Å was determined by routing the atmospheric sample flow over a catalytic reactor which removes all the ozone present. The instrument operates on a cycle of 10 s, 5 s each for the absorbed and unattenuated beam measurements. Beer's law was used to extract the ozone partial pressure using an optical absorption coefficient of 306 cm<sup>-1</sup> atm<sup>-1</sup>; the absorption tube is 71 cm long. Pressure and temperature corrections were applied to the instrument readout to derive the partial pressure of atmospheric ozone. The instrument is a commercial unit that was especially adapted for use in the U-2 experimental system. The rated sensitivity is 3 nbar partial pressure of ozone.

A ram inlet port of diameter 1 inch brings the air sample flow into the aircraft. Flexible stainless steel lines connect the instrumentation manifold to the inlet and outlet ports. Typical in-flight air flow rates are 30 STP min<sup>-1</sup> to the nitric oxide detector and 0.3 STP min<sup>-1</sup> to the ozone detector. Motorised valves in the manifold isolate the instruments from the atmosphere and from each other up to an altitude of 12 km, at which point the valves are opened by the pilot and measurements commence. Thereafter the system operation is fully automatic. The environment in the experimental bay of the aircraft during data gathering was maintained at approximately 45° F and 4 pound inch<sup>-2</sup> absolute. The true air speed of the aircraft was 400 knots.

Data from the two instruments were recorded on magnetic tape once a second and the outputs of various pressure, temperature and flow sensors were recorded every 10 s. Graphic



displays of the raw nitric oxide and ozone data were prepared from the magnetic tape. Analysis of the data was carried out from the graphic outputs.

Figure 1 shows results obtained on the flights of December 6 and December 18, 1973 at longitude 122°W and latitudes as indicated. Conversion to nitric oxide number densities was done using the measured sample air flow, calibrator flow and calibrator nitric oxide mixing ratio. The nitric oxide number densities given in the legend to Fig. 1 are average values observed at 21 km over the entire flight segment at that altitude for each date. The average nitric oxide count rates are typically 600, 750, and 1,200 counts s<sup>-1</sup> over the 3-min zero, measure and calibrate

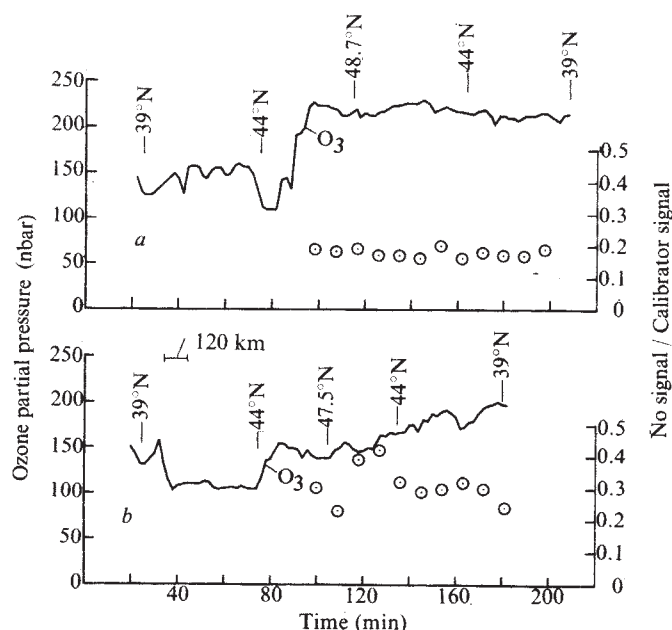


Fig. 1 Measurements *in situ* of nitric oxide and ozone from a U-2 aircraft at 18 and 21 km altitude at longitude 122° W. a, December 18, 1973,  $[\text{NO}]_{\text{ave}} = 1.3 \pm 0.6 \times 10^{-8} \text{ cm}^{-3}$ ; b, December 6, 1973,  $[\text{NO}]_{\text{ave}} = 2.2 \pm 1.1 \times 10^{-8} \text{ cm}^{-3}$ . Lower part of each curve (left) corresponds to 18 km altitude, upper part (right) to 21 km.

cycles, respectively. The statistical errors, due to the photon count rates, in the nitric oxide data points of Fig. 1 amount to only about  $\pm 6\%$ . The larger part of the error limits given for the nitric oxide number densities are from estimated uncertainties of  $\pm 5\%$  in the sample and  $\pm 20\%$  in the calibrator flow rates and  $\pm 20\%$  in the calibrator mixing ratio.

The measured loss of nitric oxide in the inlet flow lines is less than 5%; no correction has been made for this. We also measured the loss of ozone in the inlet lines in the laboratory as a function of time from the start of flow of an ozone sample through the lines. The loss is 33% of the input ozone at 30 min decreasing linearly with time to 16% at 210 min. After the appropriate correction for the measured ozone line loss, the estimated uncertainty in the ozone data is  $\pm 20\%$ , of which  $\pm 15\%$  is due to systematic errors.

Data taken at 18 km for nitric oxide are not shown. A system conditioning, indicated by changing calibrator levels, occurred during the first hour of flight. After 1 h the two calibrator levels and their ratio stabilised at values close to those observed in the laboratory before and after the flight. Modifications are in progress to correct this difficulty.

Our measurements of nitric oxide concentrations are in agreement with another chemiluminescent measurement made in the same altitude range<sup>1</sup>. But our measured concentrations are lower, by a factor between three and ten, than recently reported optical measurements<sup>2,3</sup>.

We thank M. A. Knutson and R. Danielson of the NASA Earth Research Aircraft Project for help. This work is part of a NASA program, partially supported by the Climatic Impact Assessment Program of the US Department of Transportation.

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## Temperature, fossil CO<sub>2</sub> accumulation and carbonate ion concentration of the oceanic mixed layer

SEVERAL authors<sup>1-4</sup> have recently suggested that there will be a dramatic accumulation of fossil CO<sub>2</sub> in the oceanic mixed layer by the end of this century. If these calculations are correct the predicted increases in the atmospheric partial pressure of carbon dioxide ( $p_{\text{cd}}$ ) and in the total concentration of carbon dioxide species in the mixed layer ( $C_{\text{T}}$ ) do not imply catastrophic decreases in the carbonate ion concentration<sup>1,3</sup> at 25° C (ref. 5). Two important points remain unresolved, however, and they require further clarification.

The first point concerns the relative merits of the various mixing models. The earlier calculations<sup>5</sup> raise doubts about the validity of the more pessimistic mixing models<sup>1-4</sup> because they indicate that a system reasonably close to equilibrium can only accommodate the predicted  $C_{\text{T}}$  and  $p_{\text{cd}}$  values if the carbonate alkalinity ( $CA = (\text{HCO}_3^-) + 2(\text{CO}_3^{2-})$ ) is allowed to increase. This implies either that the reaction



is not the only process involved in the assimilation of fossil CO<sub>2</sub> in the mixed layer, or that the mixed layer itself is grossly out of equilibrium with the atmosphere. Neither conclusion is consistent with the assumptions of the mixing models<sup>1-3</sup>.

A more realistic approach is to explicitly confine the assimilation process to reaction (1) so that  $CA$  remains constant and to assume a reasonable measure of equilibrium between the atmosphere and the mixed layer. The concentrations of the carbonate and bicarbonate ions in the mixed layer can then be calculated from the equations derived by Park<sup>6</sup>:

$$c_1 = (\text{CO}_2 \cdot \text{H}_2\text{O}) = \alpha p_{\text{cd}} \quad (2)$$

$$c_2 = (\text{HCO}_3^-) = (X - K_3' c_1)/4 \quad (3)$$

$$c_3 = (\text{CO}_3^{2-}) = (4CA + K_3' c_1 - X)/8 \quad (4)$$

$$\text{where } X = ((8CA + K_3' c_1) K_3' c_1)^{1/2} \quad (5)$$

and  $K_3'$  is the equilibrium constant for reaction (1). The values of  $p_{\text{cd}}$  predicted by various workers are in good agreement with one another<sup>3</sup> as they must take due account of the increases observed to the present day. Calculations based on these equations (Skirrow and Whitfield, in preparation) confirm that those models<sup>1-4</sup> which suggest that 25% of the injected fossil CO<sub>2</sub> will reside in the mixed layer, represent systems grossly out of equilibrium with the atmosphere. The model of Broecker

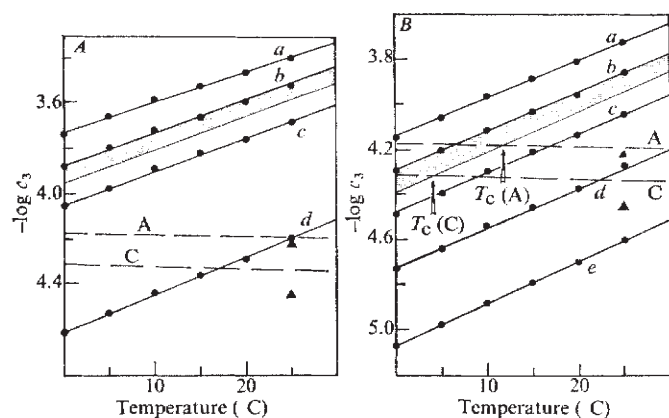


Fig. 1A, Variation of carbonate ion concentrations with temperature in 1974 when  $p_{\text{cd}} = 330 \times 10^{-6}$  atm. Solid lines, seawater values calculated from equations (2) and (4) with the carbonate alkalinities (meq  $\text{l}^{-1}$ ): a, 3.0; b, 2.5; c, 2.0; d, 1.0. Shaded area, typical oceanic range. Dashed lines, the carbonate ion concentrations in equilibrium with aragonite (A) and calcite (C) calculated from equations (8) and (7) respectively.  $\blacktriangle$ , Carbonate ion concentration in equilibrium with aragonite<sup>7</sup> (upper point) and with calcite<sup>7</sup> (lower point). B, Variation of carbonate ion concentrations with temperature in 2050 when  $p_{\text{cd}} = 996 \times 10^{-6}$  atm (ref. 3) with carbonate alkalinities (CA meq  $\text{l}^{-1}$ ): a, 3.0; b, 2.5; c, 2.0; d, 1.5, e, 1.0.  $T_c$  is the critical temperature at which the carbonate ion concentration of the sea water equals that in equilibrium with aragonite,  $T_c$  (A); or calcite,  $T_c$  (C); at the saturation point. The values indicated are for CA = 2.25 meq  $\text{l}^{-1}$ , the lowest value normally encountered in the oceans. Other features are as defined for Fig. 1A.

*et al.*<sup>7</sup>, on the other hand, gives an accurate picture of the surface waters in equilibrium with the atmosphere, and predicts that only about 5% of the accumulating fossil  $\text{CO}_2$  remains in the mixed layer. Since this layer, by definition, equilibrates fairly rapidly with the atmosphere the model defined by equations (2) to (5) should give the more realistic picture of the effect of increases in atmospheric  $\text{CO}_2$  on the chemistry of the mixed layer. This model will be used to discuss the second point: the influence of temperature and carbonate alkalinity on the concentration of carbonate ions in ocean surface waters.

So far, calculations concerned with the dissolution of calcium carbonate have either made no specific mention of the ambient temperature<sup>1-3</sup> or they have been confined to 25°C, a temperature typical of tropical surface waters<sup>5,7</sup>. This bias has arisen primarily because higher latitude waters only represent a relatively small proportion of the Earth's ocean surface. In addition the prospect of calcium carbonate dissolution naturally focuses immediate attention on the fate of the tropical coral reefs. The solubilities of both carbon dioxide and of calcium carbonate in seawater increase, however, as the temperature falls, so that temperate waters will be more sensitive to increases in the value of  $p_{\text{cd}}$ .

Since the salinity effect is small over the range typically encountered in the open oceans (30–35‰) the values of  $\alpha$  given by Murray and Riley<sup>8</sup> at a chlorinity of 19‰ (approximately 35‰ salinity) can be used to calculate  $c_1$  from equation (2) for the appropriate temperature and  $p_{\text{cd}}$ . This value can then be used in equation (4), together with the appropriate value of  $K_s'$  calculated from the data of Lyman<sup>9</sup>, to give the carbonate ion concentration at any particular temperature and carbonate alkalinity. In previous calculations<sup>5</sup> (Skirrow and Whitfield, in preparation) the carbonate alkalinity has ranged from 2.2–2.9 meq  $\text{l}^{-1}$  although the span 2.2–2.5 meq  $\text{l}^{-1}$  is more typical of the open ocean<sup>10</sup>.

The carbonate ion concentration has been calculated from equations (2) and (4) as a function of temperature and carbonate alkalinity for the present day (Fig. 1A) and for the year 2050 (Fig. 1B) when, according to Zimen and Altenhein,  $p_{\text{cd}}$  will attain its maximum value.

The concentration of carbonate ions in equilibrium with

calcite or aragonite at the saturation point can be calculated from the relationship

$$(\text{CO}_3^{2-}) = K_{\text{sp}}'/(\text{Ca}^{2+}) \quad (6)$$

where  $K_{\text{sp}}'$  is the apparent solubility product of the relevant carbonate mineral in sea water. According to Ingle *et al.*<sup>11</sup>

$$K_{\text{sp}}' (\text{Calcite}) = (1.390 - 3.096 \times 10^{-3} T^\circ \text{C}) \times S \times 10^{-8} \quad (7)$$

As the solubility product of aragonite is pure water is approximately 0.14 logarithmic units higher than that of calcite<sup>12</sup> the corresponding equation for aragonite would be  $K_{\text{sp}}' (\text{Aragonite})$

$$= (1.919 - 3.096 \times 10^{-3} T^\circ \text{C}) \times S \times 10^{-8} \quad (8)$$

Carbonate ion concentrations at saturation, derived from these equations assuming  $(\text{Ca}^{2+}) = 10 \text{ mmol l}^{-1}$ , are shown in Fig. 1A and B. Seawaters with carbonate ion concentrations lying above these lines will remain supersaturated with respect to the appropriate mineral, whereas those lying below the line will approach saturation. Although the calculations confirm earlier statements<sup>5</sup> that tropical waters will remain supersaturated throughout, they do indicate that by the year 2050 temperate waters with a mean annual temperature of 10°C (in general<sup>13</sup> those north of latitude 50°N and south of latitude 50°S) will have reached the saturation point for aragonite (Fig. 1B). The concentration of carbonate ions will not fall below this level, because the dissolution of carbonate minerals will absorb any additional carbon dioxide<sup>7</sup>:



This reaction increases both the carbonate alkalinity and the calcium ion concentration and so the system is effectively buffered. These parameters would also increase as the result of enhanced weathering of terrestrial rocks which should become noticeable as the value of  $p_{\text{cd}}$  rises in the atmosphere.

Using the data of Zimen and Altenhein<sup>9</sup> the calculations used in constructing Fig. 1B can be repeated and the critical saturation temperature ( $T_c$ , Fig. 1B) can be calculated at the end of each decade for seawaters with a particular carbonate alkalinity. All waters with a mean annual temperature less than  $T_c$  are likely to approach the saturation level for the mineral specified. The resulting curves (Fig. 2) indicate that, for CA = 2.25 meq  $\text{l}^{-1}$ , waters with mean annual temperatures greater than 5°C are likely to remain supersaturated with respect to calcite and those with temperatures greater than 12°C will remain super-

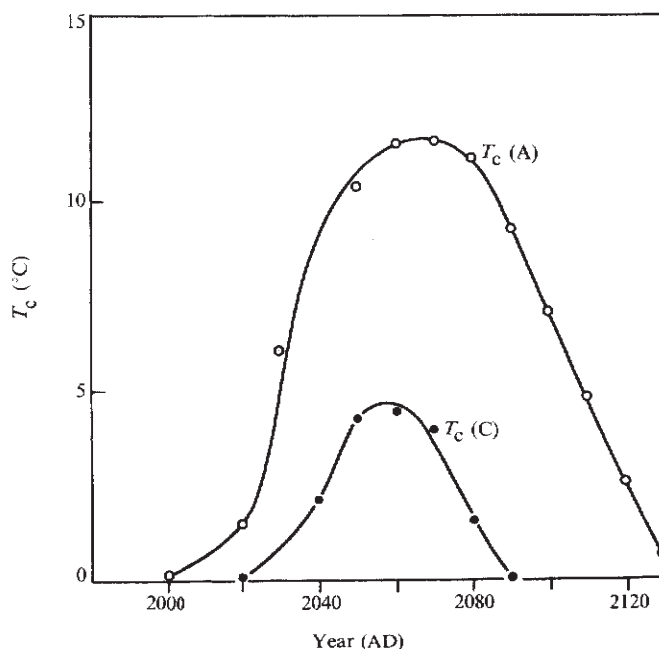


Fig. 2 The variation of the critical saturation temperature for aragonite,  $T_c$  (A); and calcite,  $T_c$  (C); over the next one and a half centuries calculated from predicted variations in  $p_{\text{cd}}$  (ref. 3) for seawater with a carbonate alkalinity of 2.25 meq  $\text{l}^{-1}$ .



saturated with respect to aragonite. The effect is transient, with the time at saturation point increasing as the temperature falls. The detailed picture is of course critically dependent on the solubilities of the various carbonate minerals. Higher<sup>14</sup> and lower<sup>7</sup> values than those given by equations (7) and (8) have been reported for the solubility of calcite and aragonite. A full analysis will become possible only when we have a clearer idea of the values of these parameters at low temperatures.

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Received March 4, 1974.

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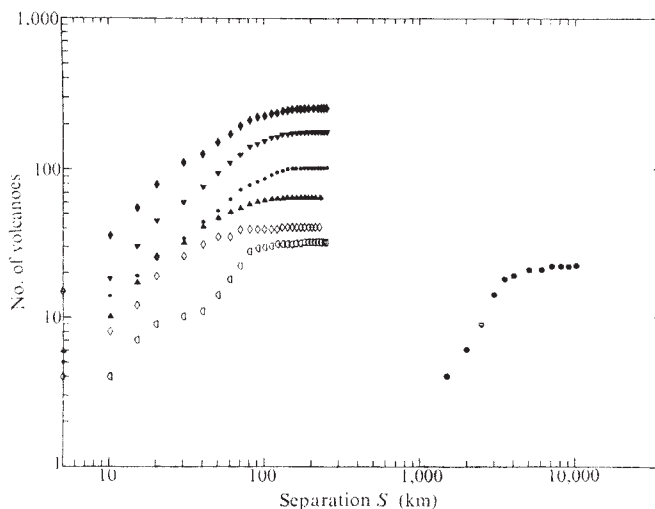
## Hot spot and trench volcano separations

We suggest that the distribution of separations between trench volcanoes located along subduction zones reflects the depth of partial melting, and that the separation distribution for hot spot volcanoes near spreading centres provides a measure of the depth of mantle convection cells.

It has been argued that frictional heating along slip zones between subducting and overriding plates provides the heat for partial melting<sup>1,2</sup>. At hot spots the suggested sources of heat are more controversial, ranging from shear heating in the asthenosphere<sup>3</sup> to more vague sources associated with deep thermal plumes originating at the core–mantle interface<sup>4</sup>. The depth of partial melting under volcanoes along slip zones is  $100 \pm 20$  km, estimated<sup>5</sup> from considerations of the distance of volcano chains behind trenches and the seismically determined dip angles of the subducting plate.

There is a break in the distribution of separations between trench volcanoes at separations of  $\sim 100$  km, and we suggest that this indicates the depth of partial melting beneath these volcanoes. For each volcano along the various trenches we have calculated the separation,  $S$ , from its nearest neighbours<sup>6–10</sup>. The cumulative distribution of the number of volcanoes with nearest neighbours within a distance  $S$  is plotted against separation (Fig. 1) for volcanoes along major trenches and for the sum of these volcanoes. These distributions all rise rather uniformly up to separations of about 100 km and then flatten, indicating that there are relatively few trench volcanoes with nearest neighbours more than 100 km away.

Assuming that the region of partial melting is a belt along the slip zone at a depth of  $\sim 100$  km and that all along this belt the magma migrates upwards along random paths to the



**Fig. 1** The cumulative distribution of separation distances for trench and hot spot volcanoes, that is, the number of volcanoes separated from their nearest neighbours by less than  $S$ . Solid diamonds and solid inverted triangles are the sums of all trench distributions (from the International Association of Volcanology and ref. 10, respectively); solid circles, Indonesia<sup>8</sup>; solid upright triangles, Kamchatka-Kuriles<sup>6,9</sup>; open diamonds, central America<sup>10</sup>; open semicircles, Aleutians<sup>10</sup>; solid circles, hot spots<sup>4</sup>.

surface to form volcanoes, it is reasonable to suggest that the separation between the volcanoes would not generally exceed the depth of the magma source.

Considering hot spot volcanoes located essentially along the oceanic ridge system<sup>4,11</sup>, we have found that the distribution of separations between each hot spot volcano and its nearest neighbour shows a similar break or flattening (Fig. 1), but at a separation of  $\sim 3,000$  km, indicating that most hot spots have nearest neighbours within 3,000 km. This distance is the same as the depth to the core–mantle boundary, where deep thermal plumes may originate<sup>4</sup>.

We propose that the lateral dimensions of mantle convection cells are also represented by the hot spot separations, rather than by ridge–trench distances<sup>12,13</sup>, and that the break in the distribution of hot spot separations at 3,000 km is evidence for both whole mantle convection and a deep thermal plume origin of hot spots. We do not suggest that the magma extruded from hot spot volcanoes comes from the core–mantle boundary. The magma source is at the top of the convective plume just under the oceanic plate, and is consistent with a break at  $< 100$  km in the distribution of separation distances<sup>16</sup> between the volcanoes which make up the Hawaiian chain of islands and guyots.

This research was financed by NASA and the National Science Foundation.

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Received January 10; revised April 1, 1974.

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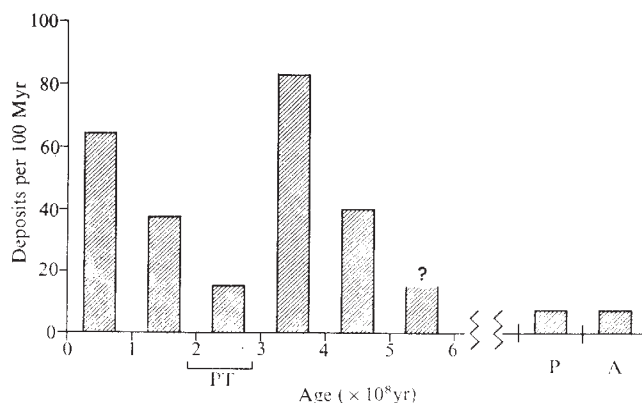


Fig. 2 Variation in the number of massive sulphide deposits per 100 Myr throughout the geological column. The Proterozoic (P) and Archaean (A) are each assumed to be 1,500 Myr long. PT, Permo-Triassic.

## Massive sulphides and plate tectonics

SEVERAL authors have considered the genesis of various ores in terms of plate tectonic theory<sup>1-8</sup>. Most have discussed various possibilities for the origin of the ore solutions of massive sulphides and, with the exception of Guild<sup>6</sup>, have stated or implied a fairly close genetic relationship between ore deposition and the processes of seafloor spreading and subduction. The nature of this relationship is, however, by no means clear and I suggest here that it may be very tenuous.

The deposits included in the term 'massive sulphide' are stratiform and wholly or largely composed of sulphides of iron with varying proportions of the sulphides of copper, lead and zinc, and minor gold and silver. Stratiform, massive oxide deposits (largely magnetite and/or haematite) are related ore types. The term includes 'volcanogenic massive sulphides', but as used here it also covers very similar ores in sedimentary or sedimentary/volcanic successions (for example, Sullivan, Rammelsberg, Cobar, and Mount Isa).

Many geologists have discussed the possibility that massive sulphide deposits may be derived from heated seawater, or from meteoric water that has become an ore solution because of a hot water-rock interaction below the surface<sup>9-11</sup>. The principal requirements of this model are: a thermal anomaly sustained for sufficient time to precipitate an ore deposit; a system allowing long term downward penetration of water (probably to depths of several kilometres) and then upwards to the surface through major fractures; the presence of a suitable depositional site, which is commonly on the sea floor<sup>7,12</sup>.

This geothermal model seems to account for the salient features of massive sulphides.

The principal host rocks to massive sulphides are volcanics of varying composition (mainly calc-alkaline), but some deposits occur in thick sediments (mainly shales and greywackes) with little or no evidence of volcanism (for example, Meggen,

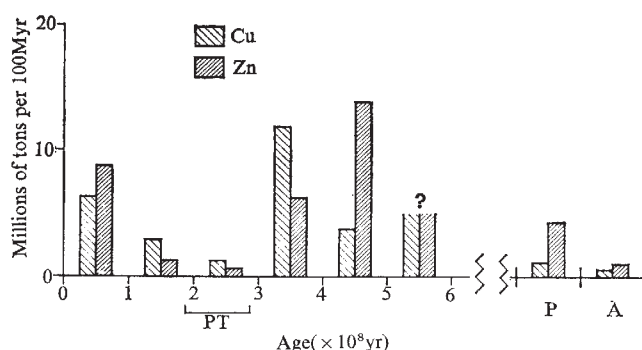


Fig. 3 Variation in the tonnages per 100 Myr of copper and zinc in massive sulphides through the geological column. The tonnages include production and reserves and are very approximate. A, Archaean; P, Proterozoic; PT, Permo-Triassic.

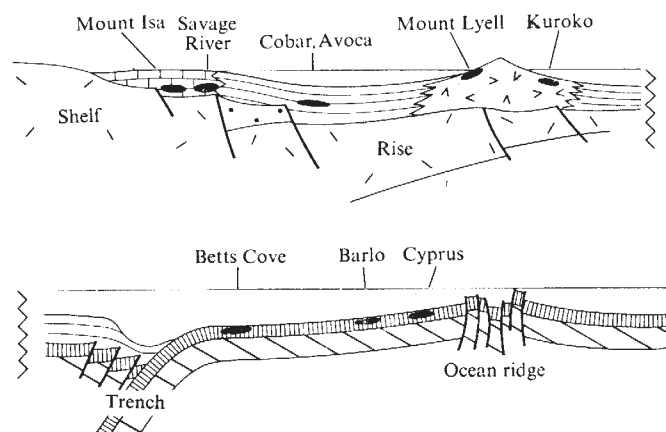


Fig. 1 Diagrammatic cross section of a continental margin to illustrate the range of tectonic environments of massive sulphides.

Rammelsberg, Cobar). There seems to be no evidence to support the statement<sup>13</sup> that massive sulphides are associated with mainly mafic volcanics. Deposits in volcanic successions do not show any correlation between the ore type (in terms of the Cu:Pb:Zn ratios) and the nature of the host rocks<sup>12</sup>. This seems to be true for all massive sulphides, except that where the host rocks are dominantly or entirely basaltic (as in Cyprus) the lead content is very low. If ore solutions derive their metal content through water-rock interactions several kilometres below the site of deposition, then correlations involving rock walls will be of little value. But a comparison between ore compositions and the nature of the rock successions for several kilometres below the deposits also showed no correlation, although the quality of the available data was poor. Copper and zinc are relatively abundant in most igneous and sedimentary rocks and the low degree of correlation between rock type and ore type is reflected in the geothermal model. Wide variations in the Cu:Zn ratio may be brought about by chemical factors during transport and at the site of deposition. Also predictable is the absence of lead ores in thick sequences of basic rocks, as these contain only a few parts per million (p.p.m.) of that metal. Another important feature of the host rocks is the lack of coeval plutons.

Massive sulphides tend to occur at distinct stratigraphic



horizons<sup>7,14</sup> which may be unconformities (Mount Lyell), sediment lenses within volcanics (Rosebery, Captains Flat), tops of volcanic sequences (Rio Tinto, Cyprus), or at changes in the composition of volcanics (Noranda area) or the nature of the volcanism (dacite domes of northern Honshu). Thus, the timing of ore deposition does not, apparently, relate to any clearly recognisable, unique event but to periods of hydrothermal activity brought about by various agencies such as faulting, pauses in volcanism, and so on.

Phanerozoic massive sulphides occur in a wide variety of tectonic environments<sup>15</sup>, particularly in environments associated with orogenic belts (Fig. 1). Most occur in calc-alkaline island arcs and Andean-type volcanic belts (Mount Morgan, Rosebery, Mount Lyell, Rio Tinto, Hopa-Murgul, Shakanai), but some occur in the flysch and shelf sequences of the continental margin (Cobar, Avoca?), or in oceanic crust in ridge and ocean floor environments (Betts Cove, Skouriotissa, Besshi?). In the Palaeozoic of south-eastern Australia most massive sulphides occur in Andean-type volcanics, and formed fairly late in the development of the volcanic belts during the 'paratectonic' phases of the main tectonic cycles<sup>16</sup>, when the volcanics were undergoing faulting, folding and granitic intrusion. Massive sulphides commonly occur in groups aligned along major features that provide deep seated plumbing systems.

Sulphide-sulphur in massive sulphides is enriched in <sup>34</sup>S relative to the meteoritic standard and generally shows only small variations within deposits. The values for some deposits may reflect mixing and re-equilibration between sulphur in magmatic water rising from depth and sulphate in seawater<sup>17,18</sup>. The same results can be achieved by mixing sulphur derived from the leaching of rocks with sulphate-bearing seawater in geothermal conditions.

Unlike several ore-types associated, and apparently genetically linked, with plutonic rocks (for example, tin-wolfram veins, nickel sulphide deposits and porphyry coppers), massive sulphides are well represented throughout the geological column (Fig. 2). Figure 2 is not very reliable because there are little adequate data from many areas, particularly from the Cambrian. The main features include the high number of deposits in the Carboniferous and Devonian, and the low number in the Permo-Triassic and the Archaean. The Carboniferous high reflects the large number of deposits in the Iberian pyrite belt, and the low value for the Precambrian (and the Cambrian?) may well be a function of the low preservation rate of these rocks<sup>19</sup>. The plot of tonnages (production plus reserves) of zinc and copper every 100 Myr (Fig. 3), though based on limited data, seems to confirm the Archaean and Permo-Triassic lows, and indicates the existence of several very large Proterozoic deposits (Sullivan, McArthur River, Mount Isa).

The principal features of massive sulphides do not conflict with, and are to some extent predicted by, the geothermal model outlined. In particular the model accounts for the lack of related plutons, the poor correlation between host rock and ore type, and the wide range of tectonic environments in which the deposits occur. The grouping along faults and the S-isotope data and overall chemical composition, are also compatible with the model.

If massive sulphides are essentially of superficial origin, with the entire ore forming process extending no more than a few km below the surface, it is difficult to see why there should be any close relationship with plate tectonic processes and related magmatism. The tendency for ore to be concentrated in marine volcanic zones in orogenic belts may simply be because such areas are the most favourable for developing convective hydrothermal systems and providing plumbing systems and depositional sites. The Permo-Triassic low in the distribution graphs may reflect the relatively low output of volcanic rocks at this time<sup>20</sup>, and the large size of the few Proterozoic deposits may be a function of relatively stable conditions in large basins. Though the model seems to account

for most of the data it does not necessarily explain the genesis of every deposit. The model needs to be tested on individual cases, possibly using isotope tracers<sup>21</sup>.

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Received December 17, 1973; revised April 9, 1974.

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## Late glacial site in the central Grampian Highlands

THE last British ice sheet probably reached its greatest extent 17,000–18,000 radiocarbon yr ago, when it covered much of the British Isles. We suggest here that this ice mass had disappeared 5,000 yr later. Significant new evidence comes from Loch Etteridge in the heart of the Grampian Highlands of Scotland (Fig. 1).

Loch Etteridge (grid ref. NN688929) lies in a deep kettle hole bordered by eskers and kame terraces at an altitude of 300 m, in upper Strathspey. The deepest accessible part of the kettle infill was sampled using a piston corer with a chamber 50 cm long and 5 cm in diameter. Seven metres of peat and gyttja overlie a typical late glacial sequence comprising organic deposits between minerogenic deposits (Fig. 2). Pollen analyses of the late glacial and basal postglacial deposits were carried out at 2.5-cm intervals. Samples for radiocarbon dating were obtained by combining material from four bores put down at the corners of a 30-cm square. This was possible because of the well defined stratigraphy, and was facilitated because the full sequence (Fig. 2) was obtained in a single chamber in each bore.

A date of  $9,405 \pm 260$  yr BP (SRR-301) was obtained for the basal 4 cm of the postglacial organic deposits. The pollen spectrum is dominated by *Artemisia*, which exceeds 30% of the total land pollen, whereas values for woody taxa are less than 10%. Immediately above the dated sample, open habitat taxa decline markedly and pollen of woody plants increases. The radiocarbon date is much later than the date of approximately 10,300 yr BP which is conventionally assigned to the end of the Loch Lomond Stadial. In

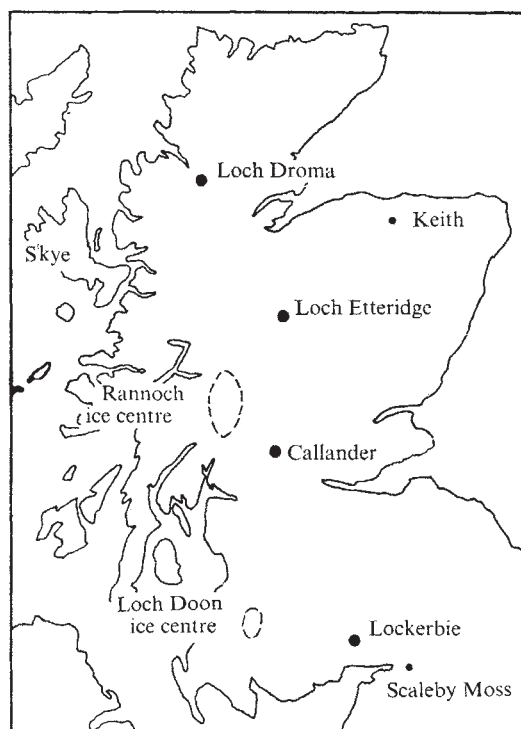


Fig. 1 Locations referred to in the text.

Skye, however, the cessation of minerogenic deposition varies considerably in different lochs, and the youngest date relating to this event is  $9,420 \pm 150$  yr BP (ref. 1). Protracted minerogenic deposition at Loch Etteridge may be related to its altitude and to its location 8 km from the margin of a plateau ice cap which covered nearly  $300 \text{ km}^2$ , and which is considered to have developed during the Loch Lomond Readvance<sup>2</sup>.

At the top of the Late Glacial interstadial deposits the pollen spectrum is characterised by woody plants, especially *Empetrum* and *Betula c.f. nana*, but *Artemisia*, Saxifragaceae and *Rumex* are also significant. In the overlying clay, pollen of woody plants declines and that of open habitat taxa increases. The top 1.5 cm of the interstadial deposits gave a date of  $10,764 \pm 120$  yr BP (SRR-302). This accords with the value of 10,800 yr BP generally accepted for the beginning of the Loch Lomond Stadial, indicated, for example, by dates from Keith in Banffshire and from Scaleby Moss in northern England<sup>3</sup>. It has been inferred that at about 10,800 yr BP minerogenic deposition (implying solifluction on surrounding slopes) began at sites in Skye<sup>1</sup>. It therefore seems that at this time temperatures fell, probably rapidly, over a large area. It does not follow, however, that the glaciers involved in the Loch Lomond Readvance began to build up at this time.

Studies of Coleoptera at sites in England, Wales and the Isle of Man, suggest that a fall of temperature began before 12,000 yr BP and continued until the end of the Late Glacial interstadial<sup>4</sup>, by which time mean July temperatures were probably  $4^\circ\text{--}5^\circ\text{C}$  below present values. The interstadial climate may have been moderately continental, with winter temperatures depressed even more below present values<sup>4</sup>. For glaciers to develop on Ben Nevis a fall of more than  $2^\circ\text{C}$  in the present mean summer temperature is required<sup>5</sup>. Combining these figures it seems that the Loch Lomond Readvance began before, perhaps well before 10,800 yr BP. It can also be inferred, in view of the varied relief of Scotland and the marked gradients of former snow lines, that glaciers began to develop at different times in different places.

At the base of the more organic interstadial deposits, the pollen spectrum is dominated by *Empetrum* (over 45%), but *Betula c.f. nana* and *Juniperus* are also significant. A sample 1.5 cm thick yielded a radiocarbon date of  $11,290 \pm 165$  yr BP (SRR-303).

The pollen spectrum at the base of the organic deposits is characterised by high values of *Rumex* (over 20% of total land pollen) with *Salix* (many of which resemble *Salix c.f. herbacea*) and Saxifragaceae also important. Values for woody plants increase progressively towards the upper part of the dated horizon.

The basal organic deposits gave a radiocarbon date of  $13,151 \pm 390$  yr BP (SRR-304). The most likely source of any error is the hard water effect, which can give apparent ages that are almost 3,000 yr too great, although the error is rarely as large as this<sup>6,7</sup>. Limestone does not crop out in the area draining to Loch Etteridge, however, and glacial erratics of limestone have not been found. No shells were found in the lake deposits. The possibility cannot be excluded, however, that inert carbon from the complex metamorphic rocks of the surrounding area was incorporated in the dated bulk sample. On the other hand, the date of  $10,764 \pm 120$  yr BP from similar (though organically richer) material higher in the sequence agrees with expectations, and there can be no hard water error here.

Because a thickness of 3–4 cm of the deposits was used for dating, the date obtained, if valid, is younger than the age of the very bottom of the organic layer. It is particularly significant that the site is a deep kettle hole, in which dead ice may have remained for hundreds (or even thousands) of years after deglaciation of the surrounding area<sup>8</sup>. Thus the basal date, if valid, is a minimum date of deglaciation.

The Loch Etteridge date is not unique in Scotland. Wood from lake deposits near Lockerbie in Dumfriesshire has

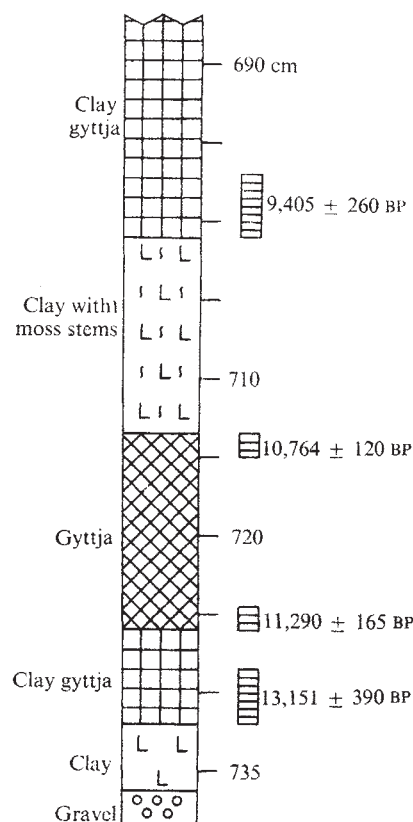


Fig. 2 Stratigraphy and radiocarbon dates at Loch Etteridge.



been dated at  $12,940 \pm 250$  yr BP (ref. 9). Lockerbie is only 60 km from the major centre of icesheet accumulation in the Southern Uplands (Loch Doon Basin). In the heart of the mountains of NW Scotland a date of  $12,810 \pm 155$  yr BP has been obtained for deposits from Loch Droma<sup>10</sup>. Near Callander the basal organic deposits in a kettle hole have provided a date of  $12,750 \pm 120$  yr BP. This site is only 45 km from the centre of the area of greatest ice sheet accumulation in the British Isles (around the Rannoch basin). The Loch Etteridge basal date adds significantly to this evidence, because the site is in the middle of the Grampian Highlands.

Together, the four dates (all of which, if valid, are minimum dates of deglaciation) show that by about 13,000 yr BP the last ice sheet had disappeared from most of Scotland. At about this time two significant, probably interrelated, events are reported to have occurred. Coleoptera and radiocarbon dates from a site in North Wales, indicate that at about 13,000 yr BP temperatures rose very rapidly, with summer temperatures becoming at least as warm as those of the present day<sup>11</sup>. Seafloor sediments indicate that deglacial warming of the North Atlantic Ocean adjacent to the British Isles occurred at about 13,500 yr BP, polar waters having retreated far to the west by 13,000 yr BP (ref. 12). In view of these profound changes we find it difficult to believe that any remnants of the British ice sheets which may have remained at about 13,000 yr BP could have survived for much longer. Total deglaciation by 12,500 yr BP seems a conservative suggestion.

The last British ice sheet, which probably reached its greatest extent at about 17,000–18,000 yr BP, had therefore wasted away by 12,500 yr BP at the latest. Subsequently, glaciers built up again in many mountain areas, and were especially extensive in the Scottish Highlands<sup>13</sup> where they began to accumulate before the climatic deterioration which occurred at about 10,800 yr BP. The glaciers probably reached their maximum extent about 10,300 yr BP, but it is not yet known how long they took to decay. The fact that minerogenic deposition, which implies solifluction and sparse vegetation, continued locally till approximately 9,400 yr BP may be significant in this context.

This research was financed by the Natural Environment Research Council. We thank Dr D. D. Harkness for the radiocarbon dating.

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Received March 6; revised April 8, 1974.

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## Late Pleistocene American obsidian tools

We have dated some of the oldest examples of obsidian use yet known in America. The obsidian originates from a new location, the Mostin site, which contains about a dozen burials and is located near the Borax Lake site in northern California. There it is thought that man lived as long ago as 10,000 yr and perhaps even at the end of the Pleistocene<sup>1</sup>.

During test excavations in the summer of 1973 human bones and obsidian artefacts<sup>2,3</sup> were found at the new Mostin site (SDA-66), located 16 km north-west of the Borax Lake site. The Mostin site ( $39^{\circ}\text{N}$ ;  $122^{\circ}50'\text{W}$ ) is situated along Kelsey Creek about 2.5 km south of Clear Lake and 5 km from the nearest obsidian source south at Mt Konocti<sup>4-6</sup> (Fig. 1). The Mostin site is now being exposed by the erosive action of Kelsey Creek which is cutting into lake sediments 6 m high deposited during an earlier higher level stage of Clear Lake<sup>7</sup>.

The samples sent to us for dating and for tracing their origin included the left fibula, left tibia and the diaphysis of the right femur of burial 4 and seven associated obsidian artefacts. The bone samples were combined and dated by the radiocarbon content of their collagen fraction according to established procedures<sup>8</sup> and yielded a date (UCLA-1795A) of  $10,260 \pm 340$  yr (ref. 9). The statistical error encompasses any deviation due to correction for isotopic fractionation of  $^{13}\text{C}/^{12}\text{C}$  in case the resident human population had been heavily dependent on freshwater fish.

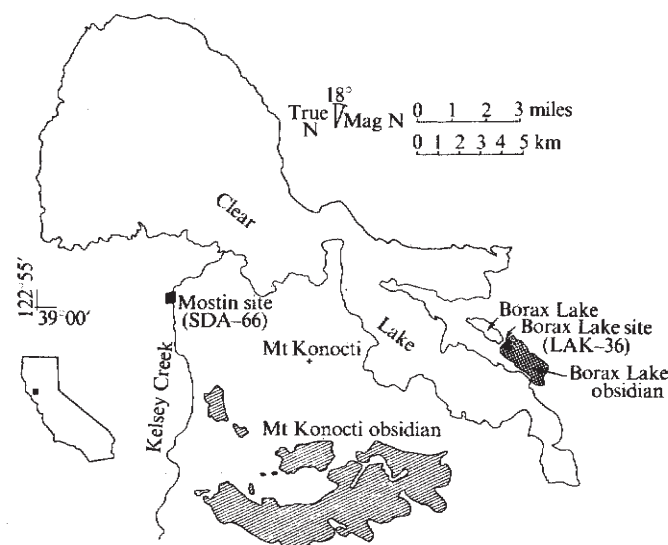


Fig. 1 Location of obsidian sources and archaeological sites, near Clear Lake, California.

The obsidian artefacts were classified into a functional typology based on their morphology and microscopic wear patterns (Table 1). Artefacts M3 (burin) and M6 (scraper) were used as tools, whereas M1 was a biface blank and M4, 5 and 7 primary decortication flakes removed from nodular cobbles of obsidian. Artefacts M1 and 6 showed fractures indicative of percussion flaking with a soft instrument from a prepared core. M2 has the appearance of a blade but its precise classification is uncertain.

All obsidian pieces were subjected to hydration rim thickness measurements and source characterisation by neutron activation and X-ray fluorescence analysis. From the differences in hydration values it became apparent that probably two types of obsidian had been utilised. Artefacts M1, 3 and 6 show a clustering of hydration rind values between 6.2 and 6.8  $\mu\text{m}$ , whereas M4, 5 and 7 are all 4.6  $\mu\text{m}$ . The appearance of artefacts 1, 3 and 6, and 4, 5 and 7 are the same respectively within each group, whereas both groups are distinctly different in visual appearance.

Table 1 Functional typology of obsidian artefacts

| Artefact sample no. | UCLA obsidian hydration no. | Hydration measurement ( $\mu\text{m}$ ) | Origin by X-ray fluorescence* | Functional typology         | Notes                                                                                          |
|---------------------|-----------------------------|-----------------------------------------|-------------------------------|-----------------------------|------------------------------------------------------------------------------------------------|
| M1                  | 3754                        | 6.4                                     | Borax Lake                    | Biface blank                | Partially finished preform                                                                     |
| M2                  | 3755                        | 5.7/12.6                                | Borax Lake                    | Blade                       | Unretouched irregular blade with a single medial ridge and convergent edges                    |
| M3                  | 3756                        | 6.2                                     | Borax Lake                    | Thick flake burin           | Appearance (and probable function) of an oblique truncation burin—a European Palaeolithic form |
| M4                  | 3757                        | 4.6                                     | Mt Konocti                    | Primary decortication flake | Broken from M7, no retouch                                                                     |
| M5                  | 3758                        | 4.6                                     | Mt Konocti                    | Primary decortication flake | No retouch                                                                                     |
| M6                  | 3759                        | 6.8                                     | Borax Lake                    | Flake scraper               | Three edges exhibit even, regular wear pattern of small scalar flakes                          |
| M7                  | 3760                        | 4.6                                     | Mt Konocti                    | Primary decortication flake | Broken from M4, no retouch                                                                     |

\* Source discrimination based on results of a ternary diagram of the intensity of the elements zirconium, rubidium and strontium.

Neutron activation analysis was too indistinct in this case to separate two suspected sources of obsidian, Mt Konocti and Borax Lake, but additional X-ray fluorescence measurements revealed by comparison with known source materials that the slower hydrating obsidian originated from Mt Konocti and the faster from the Borax Lake area.

Comparison of hydration and source data with a hydration rate curve for Borax Lake over a time span of the past 4,000 yr suggests that the rate over the entire 10,000 yr available now is at least partially exponential in nature (unpublished work of F. J. Findlow, S. P. De Atley, and J. E. Ericson). Moreover, this work shows that some two dozen obsidian artefacts found at Borax Lake may be much older than had been anticipated.

The Borax Lake assemblage also contains obsidian originating from Mt Konocti about 5 km away. The reason for this exploitation may lie in the fact that obsidians from the two sources differ in hardness: Mt Konocti material has a hardness of 500 to 600 kg mm<sup>-1</sup> whereas that from Borax Lake is 734  $\pm$  12 kg mm<sup>-1</sup>.

This work suggests that obsidian was used in Northern California at least as early as the interface of the Late Pleistocene/Holocene and the extent of early settlement may indeed have been much more extensive than hitherto thought.

We thank R. King, California State College, Sonoma for sample materials, J. T. Wasson for permission to use the neutron activation equipment at University of California, Los Angeles, J. L. Bischoff for X-ray fluorescence instruments at USC, and V. Bennett for hydration rim and C. A. Singer for functional analysis, at UCLA. This study was supported by the National Science Foundation.

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Received February 22; revised April 8, 1974.

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## BIOLOGICAL SCIENCES

### Particle containing uncleaved major head protein (P23) which is maturable into the head of phage T4

PREVIOUS results<sup>1</sup> suggested that  $\tau$ -particles produced by a phage T4 temperature-sensitive (*ts*) mutant in gene 24 can be matured into T4 phage heads after a shift to a permissive temperature (temperature shift-down). The experimental evidence which supports this hypothesis can be summarised as follows: (1) the number of  $\tau$ -particles per bacterium observed after temperature shift-down of a 24<sup>-</sup> infected culture decreases concomitantly with an increase in the number of normal T4 phage particles; (2) preshift (under nonpermissive conditions) 24<sup>-</sup> radioactively pulse-labelled cultures produce radioactively labelled normal phage after temperature shift-down; (3) after temperature shift-down, preshift radioactively labelled major head protein P23 (molecular weight 59,000) is cleaved into P23\* (molecular weight 47,500) both molecular weights determined by A. Tsugita from the amino acid composition, (personal communication)<sup>2-5</sup>.

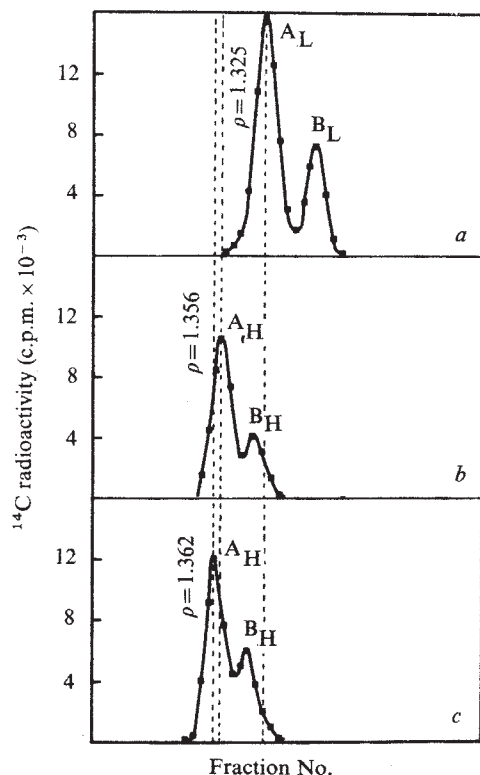
But we also know that  $\tau$ -particles produced in 21<sup>-</sup> infected bacteria cannot be further matured after temperature shift-down, although they are morphologically identical to those produced in 24<sup>-</sup> infected cultures<sup>1,6</sup>.

Here we shall demonstrate that the uncleaved P23 is organised in the form of a precursor particle, in which the later conversion of P23 to P23\* is conservative. Our complete set of experiments will be published elsewhere<sup>16</sup>. With the previous observations, these new results provide very strong evidence that 24<sup>-</sup> $\tau$ -particles are maturable into phage heads.

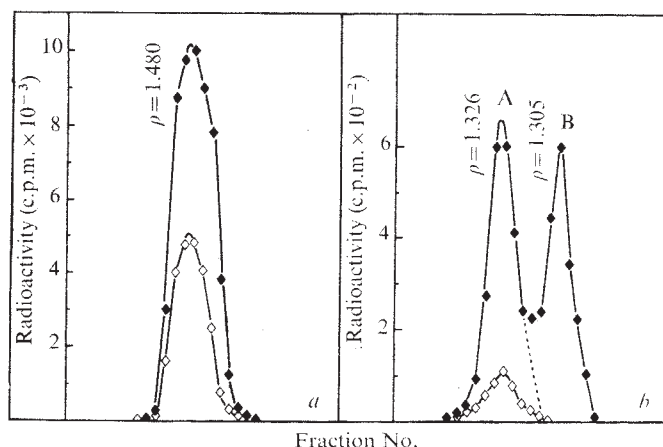


Our previous results<sup>1</sup> did not rule out the possibility that the phage capsids observed after temperature shift-down are built from subunits drawn from a pool containing subunits built before as well as after shift. (In the following we shall distinguish them as 'preshift subunits' and 'postshift subunits'.) An important part of preshift subunits could stem from the breakdown of  $\tau$ -particles (see discussion in ref. 1). If capsids of phages made after shift were composed of a mixture of preshift and postshift subunits, then a dispersive mode of transfer would have taken place. The transfer would be conservative when the preshift subunits already formed a particle which, after shift, was transformed as a whole into a capsid. We shall demonstrate (Figs 1 and 2) a true conservative transformation of a P23-capsid into a P23\*-capsid, by density labelling the preshift subunits as 'heavy' and the postshift subunits as 'light'. By supplementary radioisotope pulse labelling the fate of both types of subunits can be followed independently.

We used T4.10(amB255).24(tsL90). The tail-less heads of mutants in gene 10 have the advantage of being easily emptied by DNase treatment<sup>7</sup>. This allowed us to analyse the density of emptied heads without having to take into account the full DNA complement of a phage head and the density variability due to mixing of preshift and postshift phage tails. After a partial purification by sucrose gradient velocity sedimentation, the emptied heads were looked at in the electron microscope, their protein composition was analysed by SDS-polyacrylamide gel electrophoresis<sup>2,8</sup> and their density was measured by CsCl gradient centrifugation.



**Fig. 1** Density distribution of phage heads treated with DNase obtained in heavy medium alone, after transfer from heavy into light medium and in light medium alone. DNase treated phage heads were purified by a sucrose gradient of which the 300S fractions containing emptied head were collected. These fractions were then analysed by density equilibrium centrifugation. *a*, Emptied heads obtained from  $10^{-}$  infected cells grown in normal light medium; *b*, emptied heads obtained from  $10^{-}$ .24 $^{-}$  infected cells at 30 min after the culture was shifted from completely heavy medium at 40.5°C into normal light medium at 30°C (permissive temperature); *c*, emptied heads obtained from  $10^{-}$  infected cells grown in completely heavy medium. The explanation for the existence of two peaks is discussed in Fig. 2. Peaks A and B refer to partially emptied and completely emptied heads respectively. The subscripts L and H refer to light and heavy medium.



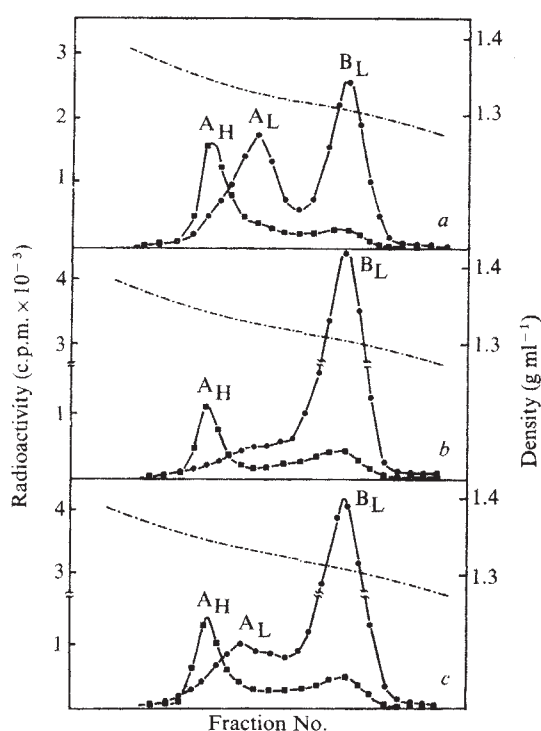
**Fig. 2** DNA content of  $10^{-}$  DNase-treated phage heads compared with the DNA content of wild-type phage. Cultures of *Escherichia coli* B<sup>E</sup> and *Escherichia coli* CR63 were infected with T4.10 (am B255). At 16 min after infection 16  $\mu$ Ci ml<sup>-1</sup> of <sup>32</sup>P and 200  $\mu$ Ci ml<sup>-1</sup> of <sup>3</sup>H-amino acids were added. At 90 min the bacteria were lysed with chloroform in the presence of DNase. The lysates were centrifuged on a 15–40% sucrose gradient for 45 min at 30,000 r.p.m. in an IEC SB 405 rotor. The 1100S peak (phage *a*) and the 300S peak (emptied heads *b*) were then layered on CsCl with density  $\rho = 1.48$  and  $\rho = 1.32$  (phage and emptied heads respectively). Radioactivity distribution of <sup>3</sup>H-labelled proteins (♦ ♦ ♦) and <sup>32</sup>P-labelled DNA (◇ ◇ ◇). Labelling conditions for phage and emptied heads were identical.

The density profile for emptied heads obtained with  $10^{-}$  infected cells in light medium is shown in Fig. 1*a*, and that of  $10^{-}$  emptied heads in heavy medium in Fig. 1*c*. In both cases there are two peaks A and B which differ in density by 0.021 g ml<sup>-1</sup>. The two peaks occur normally and are due to two types of emptied heads: those which are completely emptied (peak B<sub>L</sub>; density = 1.304 g ml<sup>-1</sup>) and those still containing about 20% of DNA (peak A<sub>L</sub>; density = 1.325 g ml<sup>-1</sup>). The incomplete removal of DNA from all of the DNase-treated  $10^{-}$  heads has been observed by Hosoda and collaborators (personal communication). We have repeated an experiment to demonstrate the presence and absence respectively of DNA in the denser and less dense types of emptied heads. The results of this experiment are shown in Fig. 2. Clearly, peak A contains DNA while peak B does not. Although both types of emptied head were nearly always found after DNase treatment, their relative amounts varied with every experiment.

From Fig. 1 we deduce that the two emptied head peaks A and B (double peak) are shifted by 0.037 g ml<sup>-1</sup> when grown in heavy medium (compare peaks A<sub>L</sub> and A<sub>H</sub>). The density profile of Fig. 1*b* was obtained from emptied heads which arose after temperature shift-down and simultaneous transfer to light medium with the double mutant 24(tsL90).10(amB255). This double peak is 0.031 g ml<sup>-1</sup> denser than the light one. This result is incompatible with a dispersive mode of transfer and suggests instead that the transfer is conservative.

It is, however, possible that the infected bacteria do not immediately synthesise structural components after shift into light medium. This would mean that preshift built subunits cannot mix with postshift newly synthesised light subunits. To test this possibility we labelled preshift built material with <sup>14</sup>C-labelled amino acids and postshift built material with <sup>3</sup>H-labelled amino acids. These results shown in Fig. 3 demonstrate that light subunits are synthesised immediately after temperature shift and that most of the <sup>14</sup>C-preshift labelled emptied heads remain in a peak which is considerably denser than the <sup>3</sup>H-labelled emptied heads. Furthermore, the <sup>14</sup>C-labelled emptied heads remain from 15 to 60 min after temperature shift.

Our previous results together with these experiments give good evidence that  $24^-$ - $\tau$ -particles can be transformed into phage heads in a conservative mode. We have, however, no proof that this  $\tau$ -particle is also a precursor in the normal pathway of phage head maturation as proposed by Simon<sup>9</sup>. Among the P23-containing particles, only the  $24^-$ - $\tau$ -particle can be further matured;  $21^-$ - $\tau$ -particles, which are morphologically indistinguishable, cannot be transformed<sup>1,6,10</sup>. The same is true for the so-called "crummy heads"<sup>11</sup> produced by *ts* mutants in gene 23<sup>16</sup>. Laemmli and Favre<sup>12</sup> have shown that in pulse-chase experiments in wild type a precursor particle called prohead I which contains predominantly uncleaved P23 and possibly P22 and IP III can be converted into a particle called prohead II, which contains essentially P23\*, P22 and IP III. Other results<sup>6,10</sup> show that the  $21^-$ - $\tau$ -particles and the prohead I particles have similar properties (S value, protein composition). These results suggest that the  $24^-$ - $\tau$ -particles may be a true precursor in the normal pathway of phage head maturation.



**Fig. 3** Density distribution of pre- and postshift labelled emptied heads of  $10^{-24^-}$  infected cells at different times after temperature shift-down. *a*, Density distributions of preshift  $^{14}\text{C}$ -labelled empty heads (■ - ■ - ■) and postshift  $^3\text{H}$ -labelled empty heads (● - ● - ●) of a sample taken 15 min after temperature shift-down; *b*, *c*, similar density distributions but of samples taken 30 and 60 min after temperature shift-down respectively. Peaks  $A_L$  and  $B_L$  refer to partially emptied and completely emptied light heads. Peak  $A_H$  refers to partially emptied heavy heads.

Assuming that these precursor particles containing P23 undergo cleavage *in situ* (that is, without breaking down and reassembling in a compartmentalised space), then the number of P23 subunits must remain identical throughout the transformation. This is highly interesting, since the overall size of the phage head is about 20% larger than that of a  $\tau$ -particle<sup>13</sup>. In this context, it is interesting to note that other results<sup>17</sup> of our laboratory indicate that the lattice constant of the capsids of giant phages<sup>14</sup> is different from that of polyheads. From previous considerations on polymorphism<sup>15</sup> it is likely that  $\tau$ -particles have the same lattice as polyheads and similarly that the giants are not different from normal phage in this respect.

We thank Drs J. Hosoda and M. Showe for suggestions and reading the manuscript.

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## Banding and chromatid separation in Chinese hamster chromosomes

In general, a chromosome exhibiting its banding pattern does not permit any internal helix to be seen, and vice versa. During studies with chromosomes of Chinese hamster cells, cultivated *in vitro*, it was possible to correlate G banding and spiral structure with the spatial relation between sister chromatids. G banding of human chromosomes was first described by Sumner *et al.*<sup>1</sup> and later was demonstrated in the Chinese hamster chromosomes by Radabli and Kriukova<sup>2</sup>. By a hypotonic treatment, the spiralisation of the chromosomes was demonstrated by Ohnuki<sup>3</sup> in man and later by Kato and Yoshida<sup>4</sup> in the Chinese hamster.

Chinese hamster fibroblasts of the DON line were grown for 2-3 d in Puck's medium supplemented with 10% calf serum and 1% glutamine, collected by trypsinisation after 5.5 h of colchicine treatment ( $10 \mu\text{g ml}^{-1}$ ), and then centrifuged at 1,000 r.p.m. for 10 min. The supernatant was replaced by a mixture of KCl (0.055 M), KSCN (0.062 M),  $\text{NaNO}_3$  (0.055 M) and  $\text{CH}_3\text{COONa}$  (0.055 M) in the proportion 4:1:2:2, which is the hypotonic saline used by Ohnuki<sup>3</sup> to demonstrate spiralisation in chromosomes. Cells were then washed, fixed for 50 min with methanol and glacial acetic acid (3:1), spread on slides according to the method of Moorhead *et al.*<sup>5</sup>, stained with 2% Giemsa R66 for 30 min, washed with tap water, dried at room temperature and mounted in DPX. Metaphase chromosomes were examined and some of them were photographed with a Zeiss  $\times 100$  Neofluar objective.



Table 1 The distribution of 106 metaphase figures

| Separation of sister chromatids                               |    | Appearance of chromatids |              |               |             | Total |
|---------------------------------------------------------------|----|--------------------------|--------------|---------------|-------------|-------|
|                                                               |    | Banded                   | Intermediate | Visible helix | Homogeneous |       |
| Separate<br>Partly<br>separate<br>Closely<br>apposed<br>Total | 0  | 0                        | 1            | 38            | 0           | 39    |
|                                                               | 0  | 0                        | 26           | 1             | 4           | 31    |
|                                                               | 36 | 36                       | 0            | 0             | 0           | 36    |
|                                                               |    | 36                       | 27           | 39            | 4           | 106   |

They showed either banding, a spiral structure, an intermediate appearance, or homogeneity without evident internal structure; the first three are exemplified in Fig. 1. It is of interest that the banding pattern appeared on the chromosomes without postfixation treatment, which is usually used for banding, and was described by Sumner *et al.*<sup>1</sup>. Within any one cell, all chromosomes were of similar appearance. It was also noted whether sister chromatids were closely opposed to each other, or were separated. If they were separated, the outside width of the whole chromosome was about 2.4  $\mu\text{m}$ , but only 0.8  $\mu\text{m}$  if the chromatids were together. Thus, the apparent separation was due to movement apart and was not wholly explicable by loss of material between the chromatids. It is interesting to compare Darlington's correlation of internal change in chromosomes with their external mechanics<sup>6</sup>, but he was concerned with the question of an internal split, whereas the results reported here relate to regional affinity for dyes. In some cells the chromatids were separated along only a part of their length. In each of 10 slides, at least 10 metaphase figures were examined and classified, the counts so obtained are presented in Table 1, which shows that closely paired chromatids always exhibited banding, and conversely, but that the internal helices became evident and the banding lost only when the chromatids separated.

A possible interpretation of this is that the manifestation of banding depends on chromosomal material which is also necessary for maintaining the close apposition of sister chromatids and which conceals the helix, but the disappear-

ance of the bands in this experiment is nevertheless also attributable to the withdrawal of interchromatid fibres. (Fig. 1b). Kato and Yoshida<sup>4</sup> tried to correlate G bands with coils by comparing the number of bands with the number of coils on the homologous chromosomes of the Chinese hamster. This correlation was only based on the constancy of the number of the coils in each individual chromosome. But they could only show the resemblance of the knobs, which are the relics of bands on the spirals, and the bands. No correlation has been shown between bands and the spirals on the chromosomes of the Chinese hamster. Ford *et al.*<sup>7</sup>, by using a modified whole-mount method, observed the spiral structure of human chromosomes distinctly, and found that the number of turns diminishes as mitosis proceeds. They concluded that the number of turns was not constant but varies with the preparation and doubted if the number of gyres would be of any value for the identification of the individual chromosomes, as was proposed by Ohnuki<sup>3</sup>. The diminution of the gyres in number, during mitosis, would necessitate the unwinding of the chromatids around their axis, which could lead to partial or complete disappearance of the bands. (Fig. 1c).

I thank Dr J. M. N. Boss for reading the manuscript.

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Received February 11, 1974.

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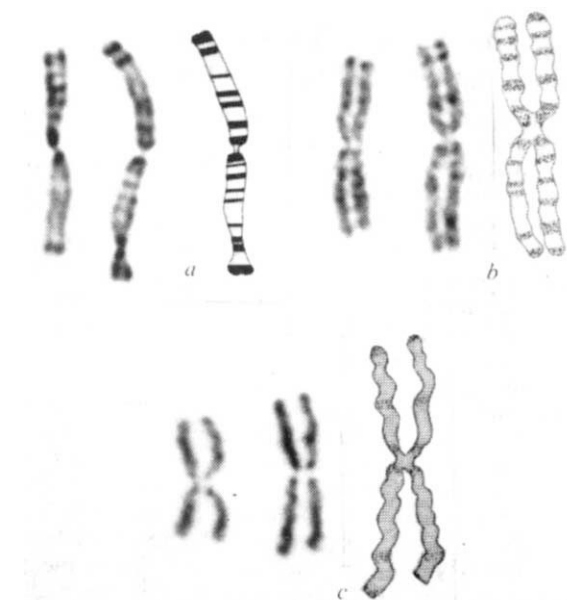


Fig. 1 Chinese hamster chromosomes treated before fixation with the hypotonic saline described in the text and without any postfixation treatment were stained with Giemsa. The chromosomes No. 1 of each group with their diagrammatic representations are shown ( $\times 4200$ ) a, Banded chromosomes; b, the chromosome intermediate in appearance between a and c; c, the chromosomes show spiralization without apparent banding.

## Virus-like particles induced in guinea pig cells by 5-bromo-2'-deoxyuridine are morphologically similar to murine B-type virus

VIRUS-LIKE particles in tissues of leukaemic and normal guinea pigs have been demonstrated repeatedly<sup>1-5</sup>. Tumour cells contain intracisternal particles resembling mouse intracisternal A particles<sup>3</sup>, and extracellular virus-like particles are found in tissues of leukaemic guinea pigs. To the best of our knowledge, a convincing micrograph of a virus-like particle budding from the surface of a guinea pig tumour cell (*in vivo*) has never been published. In a separate study (J.E.D. and K.P., in preparation), it has been concluded that

the guinea pig leukaemia virus<sup>1-3</sup> results from the release of intracisternal A particles from dying tumour cells.

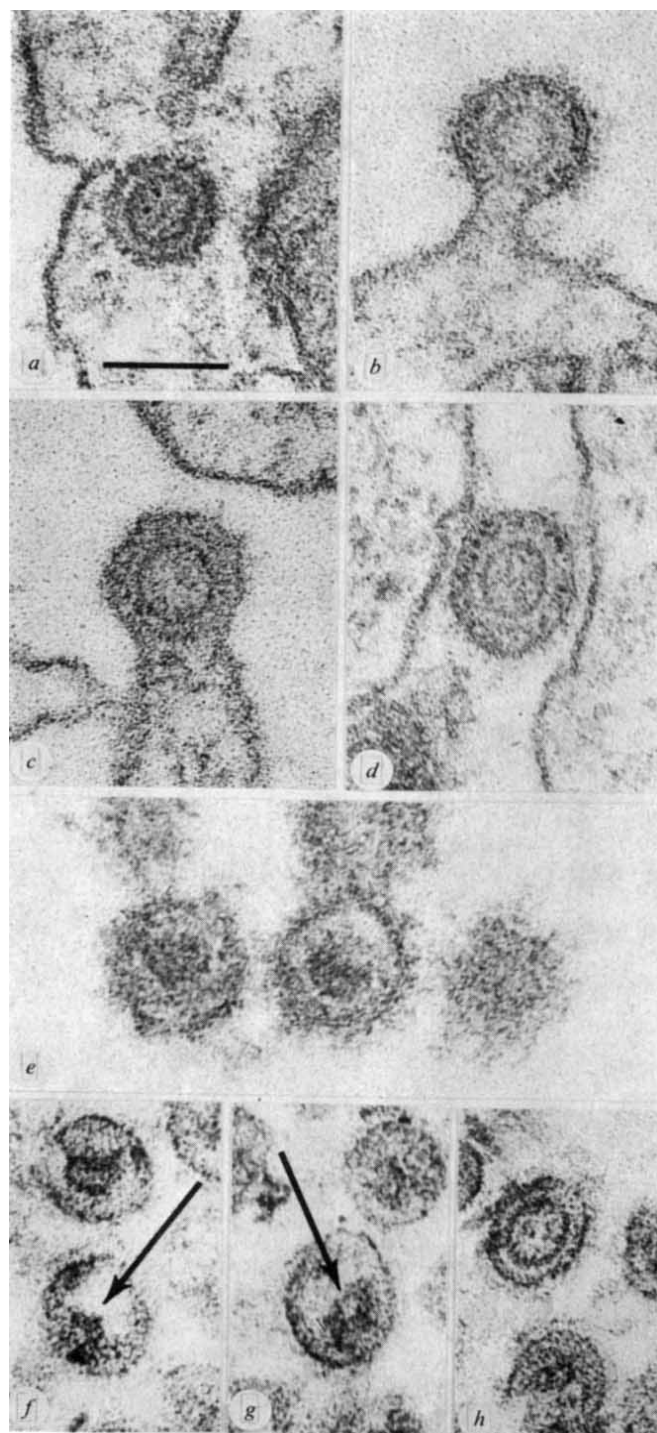
Recently, several groups have induced virus-like particles in tissue cultures of a wide variety of normal and neoplastic guinea pig cells using 5-bromo-2'-deoxyuridine (BrdU) (refs 6-9). Guinea pig cells treated with BrdU contain intracytoplasmic A particles<sup>7,8</sup> and produce extracellular virus-like particles of uncertain morphology. These extracellular particles are formed by a budding process, but it is difficult to determine from published micrographs whether these particles and buds are characteristic of C-type particles.

The categorisation of viruses found in mouse neoplasms as A, B, C, and D, first proposed by Bernhard<sup>10</sup>, and later expanded to include two types of A particles<sup>11</sup>, has recently been reviewed<sup>12,13</sup>. Intracisternal A particles bud from the rough endoplasmic reticulum into cisternae and seem to be unrelated to any other type of virus-like particles. Intracytoplasmic A particles, more homogeneous in size than intracisternal A particles, occur in the cytoplasmic matrix of the cell, and, in some cases, become the core of B particles. The intracytoplasmic A particles are not precursors of C-type particles. B particles are associated with mouse mammary tumours and can be distinguished from C-type particles by the morphology of the buds, the presence of relatively long spikes on negatively stained particles, and eccentric nucleoids in some of the mature particles. A-type particles are always intracellular, while B and C particles are extracellular.

Virus particles found in mice and other animals should be identified carefully because structural differences may represent important biochemical and genetic differences. In the case of the guinea pig, the virus-like particles induced by BrdU have alternatively been classified as C-type<sup>6,7,9</sup>, or as guinea pig leukaemia virus-like<sup>8</sup>. In the study reported here, various cell lines, including tumour cells cultured from leukaemic strain 2 guinea pigs, normal guinea pig embryo cells derived from strain NIH, and 3-methylcholanthrene and benzo[a] pyrene-treated normal guinea pig embryo cells, have been examined for virus-like particles after induction for 5 d with 40  $\mu\text{g ml}^{-1}$  of BrdU. Regardless of the source of the cells, virus particles with a morphology similar to that of the murine B-type virus, as well as intracytoplasmic A particles were the only types found. Although extracellular particles were observed frequently, budding particles were relatively rare (approximately one bud per fifty cell profiles). Figure 1 shows selected examples of thin sections of virus-like particles found in BrdU-treated guinea pig cells. Figure 1a shows an intracytoplasmic A particle; both electron dense shells are of approximately equal density. This is also true of the budding particles shown in Fig. 1b and c. At no time have budding particles been observed that failed to contain a completely formed nucleoid. Figure 1f and g illustrate eccentric nucleoids in particles occurring in a pellet of purified induced virus, while Fig. 1h shows the nucleoid of an immature particle in the same preparation. Because of the density of this pellet, osmium did not penetrate well, and the viral membranes were not well preserved. This had no effect on the nucleoids, which were adequately prefixed with glutaraldehyde. If Fig. 1a and h are compared, it can be seen that the intracytoplasmic A particle is identical to the nucleoid of a purified extracellular immature particle.

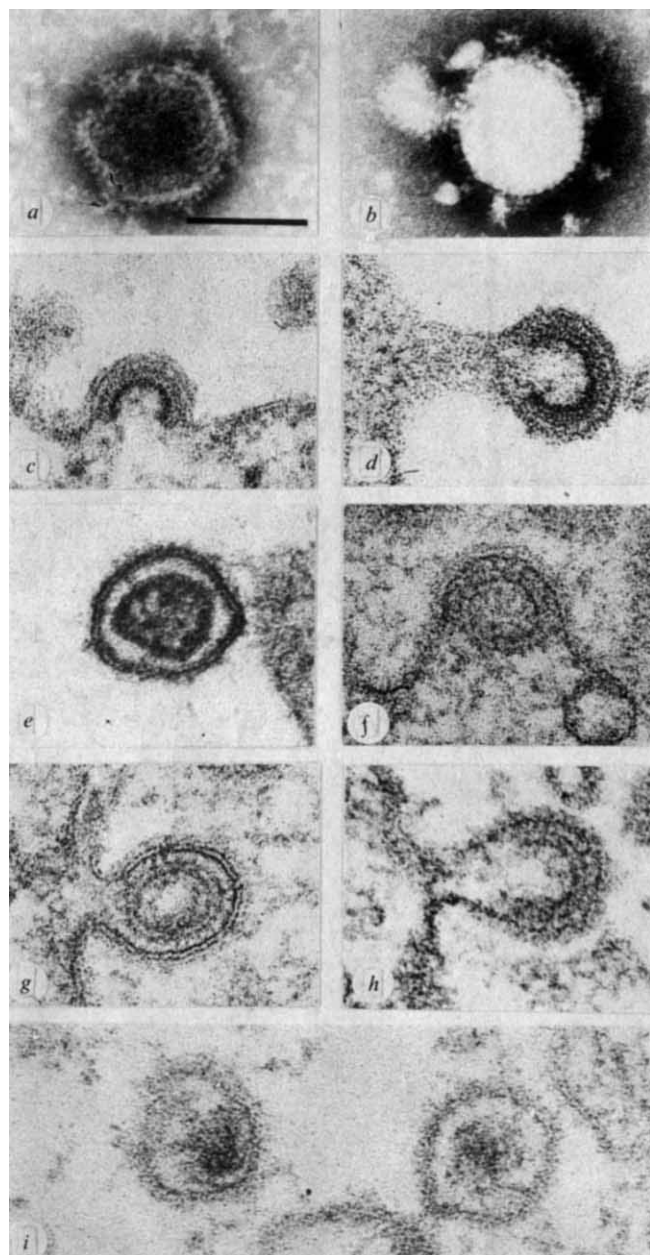
Figure 2 presents micrographs of negatively stained guinea pig particles induced by BrdU, and for comparison with Fig. 1, illustrations of several stages in the development of murine B and C particles. The presence of intracytoplasmic A particles, the ultrastructural characteristics of budding particles, and the presence of eccentric nucleoids in mature extracellular particles are criteria used to define murine type B particles. A further criterion is the presence of spikes in negatively stained particles. Although C-type particles also have spikes<sup>14</sup>, they are short and difficult to

visualise. The spikes present on the induced guinea pig particles and shown in Fig. 2a and b, most nearly resemble those found on B-type particles.



**Fig. 1** Virus-like particles induced in guinea pig cells by BrdU. Samples were fixed *in situ* with 3% glutaraldehyde in Millonig's buffer, scraped and pelleted, and post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 2 h. After dehydration in ethanol and propylene oxide, the pellets were embedded in Epon-Araldite. Thin sections were stained with uranyl acetate and lead citrate and examined at 80 kV in a Siemens 101 equipped with a 50  $\mu\text{m}$  objective aperture. The bar represents 100 nm, and all figures are at the same magnification (approximately 160,000). a, Intracytoplasmic A particle; b, c, budding particles; d, an apparently extracellular immature particle; e, extracellular mature particles; f, g, particles occurring in pellets of purified induced virus. Note the eccentric nucleoids (arrows); h, nucleoid of immature particle in the same pellet shown in f and g.





**Fig. 2** *a, b*, BrdU-induced guinea pig particles, negatively stained with 0.5% neutral phosphotungstic acid following brief fixation with 3% glutaraldehyde; *c, d*, buds of murine C-type particles (*c*, Rauscher leukaemia virus in human embryo kidney cells; *d*, the C-type particle present in L cells); *e*, mature C-type particle (RLV in HEK cells); *f, g, h*, buds of mouse mammary tumour virus (B-type virus, in mammary tumour); *i*, mature murine B-type particles (in mammary tumour). The bar represents 100 nm; all figures are at the same magnification (approximately 160,000).

Figure 2*c* and *d* illustrate buds of murine C-type particles, and show how the inner layers form concomitantly with the bud. The electron density of the inner component is significantly greater than that of the intermediate layer. Figure 2*e* shows the centrally positioned nucleoid of a mature C-type particle. Buds of murine B-type particles may either contain preformed nucleoids (intracytoplasmic A particles; Fig. 2*f* and *g*) or the nucleoid may form in synchrony with the bud (Fig. 2*h*). In either case the electron densities of the inner component and the intermediate layer are approximately equal. This is essentially what is seen in the induced guinea pig particles, although buds form only by incorporating intracytoplasmic A par-

ticles. Thin sections of mature murine B-type particles may exhibit either eccentric or central nucleoids (Fig. 2*i*), although eccentric nucleoids usually predominate. In the induced guinea pig particles, eccentric nucleoids are less frequent, and are only very noticeable in older preparations, such as the purified pellet illustrated in Fig. 1*f* and *g*.

The particles induced in guinea pig cells resemble the murine B-type particle in several important ways. Although the induced guinea pig particles may not be morphologically identical to murine B-type particles, it is evident that they are not typical C-type particles, as has been reported<sup>6,7,9</sup>.

There is a clear need for caution in categorising newly observed virus-like particles. Without high resolution micrographs of budding particles, it is extremely difficult to characterise accurately oncornavirus-like particles. Efforts are now under way to analyse biochemically particles and induced virus and the guinea pig leukaemia particles and to test their biological activities. If the preliminary diagnosis of this particle as a B-like virus is substantiated, the phenotype of a C-type virus genome has not yet been demonstrated in guinea pigs.

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Received January 14; revised April 4, 1974.

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## Cancer, autoimmunity and IgA-deficiency related by a common antigen-antibody system

WORKERS<sup>1-5</sup> have reported IgG-precipitating antibodies against predominantly high molecular weight ruminant immunoglobulins to be present in the sera of certain patients with IgA deficiencies. The most frequently identified antigen is bovine IgM but we have also found a high frequency of precipitating antibodies to bovine associated mucoprotein (BAMP) (Table 1). Immunochemical studies have demonstrated that the antibodies to these two proteins are entirely distinct and that antibodies to BAMP in human sera can only precipitate with a fraction of the antigenic determinants detected by antisera raised in rabbits against BAMP.

Bovine associated mucoprotein is a constituent of the fat globule membrane which is formed by vesicular secretion from

the alveolar epithelial cells of the bovine mammary gland. Jackson *et al.*<sup>6</sup> determined a molecular weight of 123,000 for BAMP by analytical ultracentrifugation, although lyophilised and dissolved BAMP elutes in the void volume from Sephadex G-200. BAMP contains 25% carbohydrate of which one-fifth is sialic acid, probably as a terminal residue<sup>6</sup>.

Because of the reported occurrence of autoantibodies in IgA-deficient patients<sup>7,8</sup> and because of the epithelial origin of BAMP, routine indirect immunofluorescence screening of human biopsy material was undertaken to determine whether human or rabbit antibodies to BAMP could react with human epithelial tissue. The results of this study are summarised in Table 2 and representative data shown in Fig. 1. Characteristics of the homologous system are the fluorescence of the entire cell membrane of the secretory epithelial cells, and in the mammary gland, an accumulation of BAMP on the secretory surface (luminal) of these cells (Fig. 1a). All normal human biopsies were negative but striking cell membrane fluorescence was seen on all diagnosed human epithelial cell tumours, 12% of the renal transplant biopsies, many foetal epithelial cell membranes and in systemic lupus erythematosus (SLE) skin biopsies. The positive human cells (Fig. 1b-d) were always epithelial and showed the same characteristic 'rim' fluorescence seen in bovine tissues (Fig. 1a). When the sera from patients with precipitating antibodies to BAMP were tested on sections of human mammary carcinoma or normal bovine tissue in the indirect assay, positive fluorescence was obtained with both human and bovine tissues although it was markedly more pronounced with the human tissue.

The specificity of the fluorescence for a specific antigen related to BAMP on human and bovine tissues was confirmed by four

different inhibition experiments.

The occurrence of positive pathological biopsies prompted our investigations of serum from these patients. Of those sera available, the correlation between the presence of serum antibodies to BAMP and BAMP-positive biopsies in patients with SLE was (2/2), mammary carcinoma (3/3), melanoma (1/3) and positive kidney biopsies (1/5). The occurrence of precipitating antibodies in the sera of patients suffering from similar disorders as those with BAMP-positive biopsies, sera from patients with related disorders and normal serum donors is summarised in Table 1.

Data were obtained by immunodiffusion assays with or without the addition of polyethylene glycol (PEG)<sup>9</sup>. All positive precipitin reactions could also be detected in the absence of PEG using cross-immunoelectrophoresis. No evidence was obtained for heterogeneity of antibodies to BAMP among patients with different disorders. The greatest apparent amount of precipitating antibodies occurred in patients with mammary carcinoma.

No clear-cut evidence for a BAMP-related tumour antigen in the sera of patients was obtained by: (1) precipitation in agar gel using rabbit antiserum to BAMP and (2) inhibition of capillary agglutination<sup>10</sup>. The sensitivity of the latter was 8 ng of BAMP.

The data presented here are the first to relate previous clinical and certain experimental evidences which correlate IgA-deficiency, incidence of neoplasms, transplantation and autoimmunity through a common immunochemical system—BAMP and anti-BAMP. The best interpretation of our data suggests that BAMP is a bovine protein that is antigenically related to a carcinoembryonic antigen in man. Hence, BAMP can be called a phylo-carcinoembryonic antigen. In

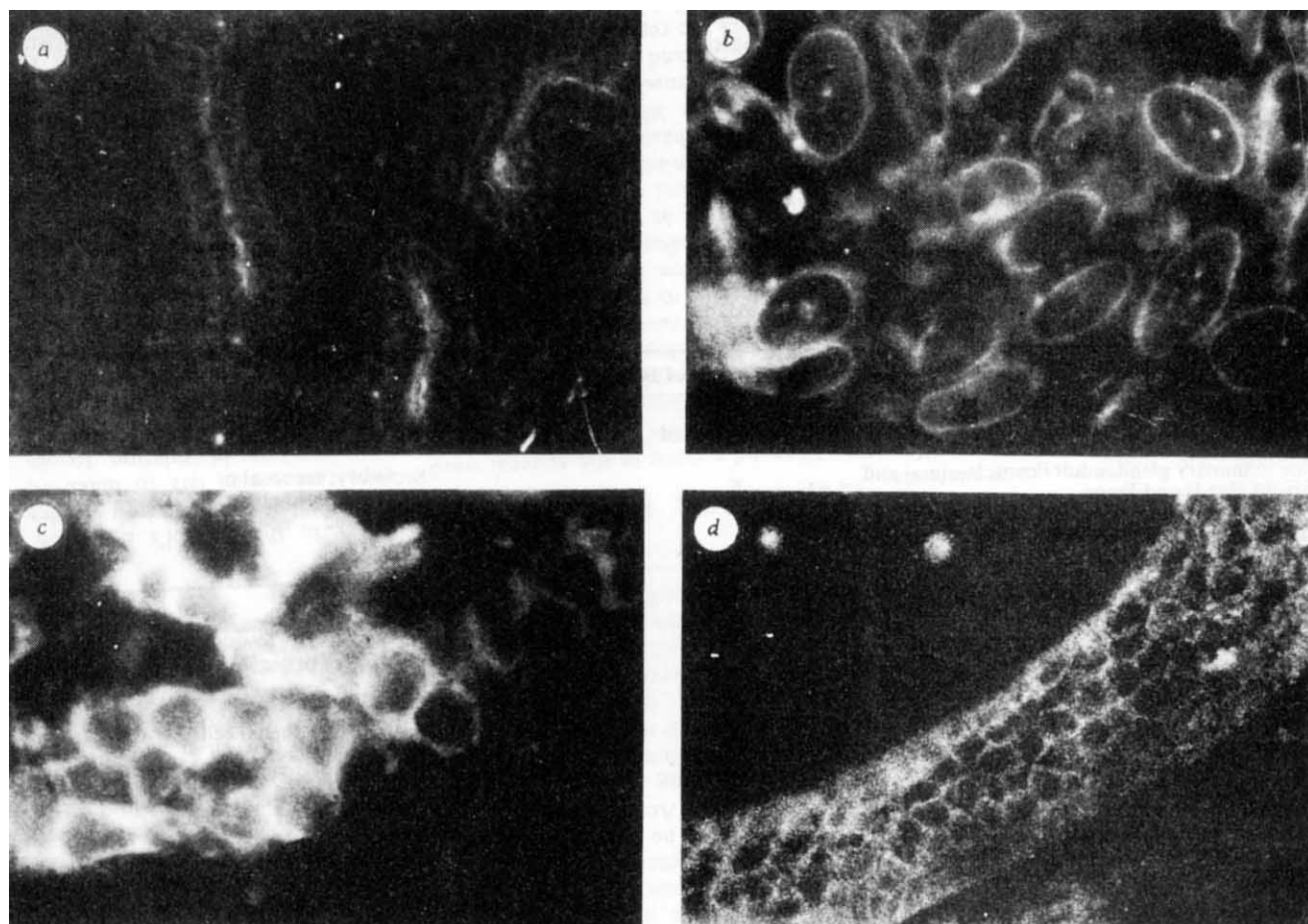


Fig. 1 Indirect fluorescence localisation. *a*, Bovine mammary gland treated with rabbit anti-BAMP (homologous system); *b*, human optic melanoma treated with rabbit-anti-BAMP; *c*, human mammary carcinoma treated with rabbit-anti-BAMP; *d*, human SLE skin biopsy treated with rabbit anti-BAMP.



**Table 1** Occurrence of precipitating antibodies to BAMP

|                                          | No.<br>tested | No.<br>positive | Group<br>% |
|------------------------------------------|---------------|-----------------|------------|
| IgA deficiencies                         |               |                 | 75         |
| Antibodies to cow's milk immunoglobulins | 11            | 8               |            |
| Nasal-pharyngeal carcinoma               | 1             | 1               |            |
| Autoimmune phenomenon                    |               |                 | 90         |
| Systemic lupus erythematosus             | 9             | 8               |            |
| Rheumatoid arthritis                     | 1             | 1               |            |
| All cancers                              |               |                 | 45         |
| Nasal-pharyngeal carcinoma               | 5             | 2               |            |
| Mammary carcinoma                        | 3             | 3               |            |
| Optic melanoma                           | 6             | 1               |            |
| Metastatic islet cell tumour             | 1             | 1               |            |
| Prostatic cancer                         | 1             | 1               |            |
| Adenocarcinoma                           | 6             | 2               |            |
| All other pathologies                    |               |                 | 22         |
| Serum from kidney patients               | 5             | 1               |            |
| Gastritis                                | 2             | 1               |            |
| Crohn's disease                          | 8             | 2               |            |
| Alcohol liver disease                    | 3             | 0               |            |
| Normal blood                             | 25            | 2               | 8          |

contrast to the previously described carcinoembryonic antigen (CEA) and  $\alpha$ -foetoprotein (AFP), the diagnostic feature of our system is the occurrence of precipitating antibodies to BAMP, whereas the reverse is true for CEA and AFP. The absence of BAMP on 127 normal biopsies and seven lymphomas and its presence on all diagnosed epithelial cell neoplasms, argues against the possibility of BAMP being a histocompatibility antigen.

The glycoprotein nature of BAMP is not unlike previously described tumour antigens and the immunochemical and physicochemical properties of BAMP are similar to those reported for CEA<sup>11-13</sup>.

Our findings show that anti-BAMP also occurs in conditions not generally regarded as neoplastic. SLE and rheumatoid arthritis (RA) are characterised by autoimmune phenomena and share certain clinical and pathological features<sup>14</sup>. IgA deficiency is not uncommon in patients with SLE<sup>15</sup> and one-third of the BAMP-positive SLE patients in this study had IgA levels lower than 70 mg%. The occurrence of precipitating antibodies to a variety of undefined homologous and heterologous antigens has also been reported in SLE and RA<sup>16</sup>. A correlation between IgA deficiency and autoimmunity has been reported<sup>17</sup> and the association between thymus function and autoimmune disease seems genuine<sup>18</sup>. The presence of BAMP and anti-BAMP in

renal transplant patients is not inconsistent with the reported correlation between neoplastic tissue and transplantation<sup>19</sup> or with reports of heterophile antibodies in transplant patients<sup>20</sup>.

The data presented here also offer a new interpretation of reports on the occurrence of antibodies to ruminant immunoglobulins in patients with IgA deficiency<sup>1-5</sup>. The presence of these antibodies has generally been ascribed to the greater absorption of macromolecules by the digestive tract of such patients. The reports that IgA-deficient patients also have autoantibodies to human IgA and human IgM<sup>7,8</sup> instead suggests that the occurrence of antiruminant antibodies could be a reflection of an autoimmune phenomenon or immune surveillance defect. In this study, (1) the failure of human sera to precipitate with only a portion of BAMP detected by anti-rabbit BAMP and (2) the greater degree of fluorescence of human carcinoma tissue compared with bovine mammary tissue obtained using human antibodies to BAMP, both suggest that the antibodies in human serum were formed in response to the human BAMP-related antigen and only cross react with BAMP. An antigen has been partially purified from a mammary carcinoma which cross reacts with BAMP and the serum of this patient contains antibodies to the human antigen.

Cell-mediated immunity is generally regarded as most important in the body's elimination of neoplastic tissue<sup>21</sup> and the specificity control at the T-cell level has been suggested to be of great importance in the prevention of autoimmune responses<sup>15,22</sup>. Reports have suggested that complete development of the IgA system depends on a functioning thymus<sup>23-25</sup>. From this, the occurrence of BAMP or anti-BAMP may be correlated with pathological conditions in which a thymic malfunction is probable.

We thank Drs Arthur Ammann, Newton Hyslop, jun., Ira Wong, William Bonney and George Pennick for the sera and biopsy material used in this study. We also thank B. Sue Newman for technical assistance and Renate Brodeur for assistance in preparation of the manuscript.

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**Table 2** Occurrence of BAMP-positive biopsies

| Nature of tissue                                                                                                                                    | No.<br>tested | No.<br>positive | Localisation<br>site                                     | Relative<br>intensity |
|-----------------------------------------------------------------------------------------------------------------------------------------------------|---------------|-----------------|----------------------------------------------------------|-----------------------|
| Bovine mammary gland, adult ileum, lacrimal and parotid glands and lungs                                                                            | 18            | 18              | Secretory, mucosal or glandular epithelial cell membrane | ****                  |
| Normal human skin, lung, ileum, colon, liver, oesophagus, adrenal and mammary gland                                                                 | 127           | 0               |                                                          |                       |
| Human foetal ileum, colon, prostate, kidney, thymus and tonsil                                                                                      | 7             | 6               | Epithelial cell membrane                                 | **                    |
| Human foetal pharynx, lip, tongue, skin, lung, oesophagus, stomach, brain, spleen, spinal cord, muscle, testes, placenta, umbilical stump and liver | 20            | 0               |                                                          |                       |
| All diagnosed epithelial cell cancer                                                                                                                |               |                 |                                                          |                       |
| Human breast carcinoma                                                                                                                              | 3             | 3               | Secretory epithelial cell membrane                       | ***                   |
| Human optic melanoma                                                                                                                                | 3             | 3               | Cell membrane                                            | ***                   |
| Human retinal balstoma                                                                                                                              | 1             | 1               | Cell membrane                                            | ***                   |
| Thyroid carcinoma                                                                                                                                   | 1             | 1               | Small epithelial membrane                                | ***                   |
| All other                                                                                                                                           |               |                 |                                                          |                       |
| Human renal transplants                                                                                                                             | 39            | 5               | Tubular epithelial cell membrane                         | ***                   |
| Skin from SLE patients                                                                                                                              | 2             | 2               | Epithelial cell membrane                                 | ***                   |
| Human prostatic tumour                                                                                                                              | 1             | 0               |                                                          |                       |
| Various lymphomas                                                                                                                                   | 7             | 0               |                                                          |                       |
| Human adenocarcinoma                                                                                                                                | 2             | 1               | Epithelial cell membrane                                 | **                    |
| Normal mouse tissue (epithelial)                                                                                                                    | 12            | 0               |                                                          |                       |
| Normal rat tissue (epithelial)                                                                                                                      | 23            | 0               |                                                          |                       |

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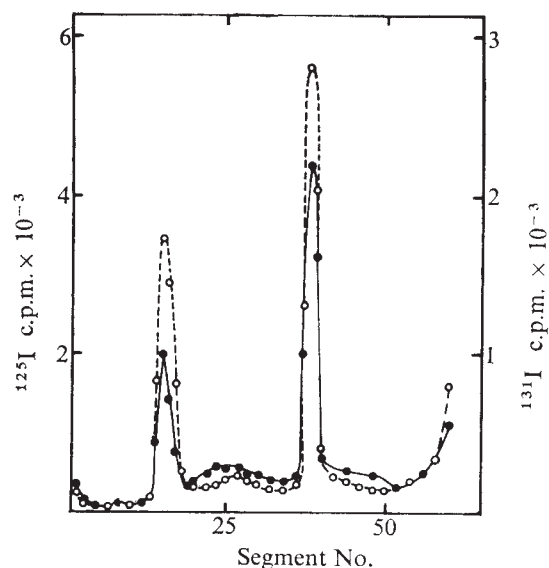


Fig. 1 SDS-polyacrylamide gel electrophoresis of immune complexes between anti-H-2D<sup>a</sup> and a <sup>131</sup>I-labelled H-2 alloantigen preparation and between anti-H-2K<sup>a</sup> and <sup>125</sup>I-labelled H-2 alloantigen. The H-2 containing materials were radioactively labelled with iodine by the chloramine T procedure<sup>10</sup>. The immune complexes were isolated by gel chromatography on Sephadex G-200. Gels with an acrylamide concentration of 15% prepared as described by Neville<sup>11</sup> were used. After completion of the electrophoretic separation the gel was divided into segments of 2 mm width and counted for radioactivity in a  $\gamma$ -well type scintillation counter. ●—●, <sup>125</sup>I; ○—○, <sup>131</sup>I.

## Subunit structure of H-2 alloantigens

HISTOCOMPATIBILITY antigens carry the immunological determinants involved in tissue transplant rejection<sup>1</sup>. Recent evidence has shown that papain-solubilised HL-A antigens are composed of two polypeptide chains<sup>2,3</sup>. The smaller of the solubilised HL-A polypeptide chains has been shown<sup>4,5</sup> to be identical to  $\beta_2$ -microglobulin and it has also been shown that  $\beta_2$ -microglobulin is physically linked to the HL-A antigens on the cell surface<sup>6-9</sup>. These findings raised the question of whether the major transplantation antigens from other species are composed of subunits. This communication shows that mouse H-2 alloantigens are linked to a small polypeptide chain which is homologous with human  $\beta_2$ -microglobulin.

Papain-solubilised H-2 alloantigens were isolated from 600 A/J spleens essentially as described for HL-A antigens<sup>4</sup>. To achieve further purification, part of the alloantigen containing material was labelled with <sup>125</sup>I and <sup>131</sup>I (ref. 10) and incubated separately with H-2K<sup>a</sup> and H-2D<sup>a</sup> alloantisera. The resulting immune complexes were isolated by gel chromatography and subjected to SDS-polyacrylamide gel electrophoresis<sup>11</sup>. From each incubation, only two radioactive bands were apparent after electrophoresis (Fig. 1). Using protein standards of known molecular weight it could be calculated that the two radioactive zones exhibited molecular weights of about 12,000 and 40,000, respectively. Control experiments with normal mouse serum from A/J mice did not give any <sup>125</sup>I- or <sup>131</sup>I-labelled material appearing in the position of the immune complexes on gel chromatography. Thus, we conclude that highly purified papain-solubilised H-2 alloantigens are composed of two polypeptide chains, irrespective of whether they are coded for by the K or the D end of the histocompatibility locus.

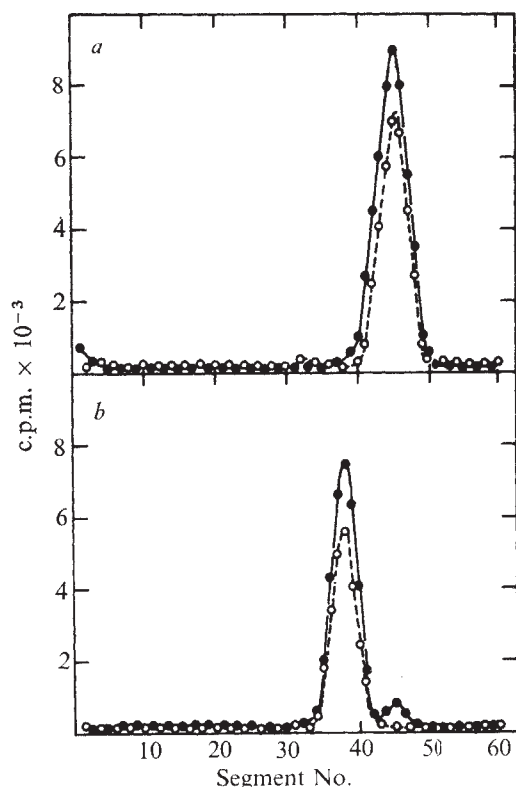
An antiserum against human  $\beta_2$ -microglobulin was incubated with a fraction containing <sup>125</sup>I-labelled H-2 alloantigen. The resulting immune complex was isolated and subjected to SDS-polyacrylamide gel electrophoresis. The electrophoretic profile revealed only the presence of the small H-2 alloantigen polypeptide chain which had migrated exactly as human  $\beta_2$ -microglobulin. We have shown that the antiserum used dissociates  $\beta_2$ -microglobulin from the larger HL-A chain<sup>4</sup> and it seems to have the same effect with H-2 antigens. This result strongly suggests that the small H-2 alloantigenic polypeptide chain is highly homologous with human  $\beta_2$ -microglobulin.

The two H-2 polypeptide chains were separated from each other by gel chromatography in 3 M KCl. The smaller polypeptide chain, labelled with <sup>125</sup>I, and <sup>131</sup>I-labelled human  $\beta_2$ -microglobulin were subjected to acid-urea starch gel electrophoresis<sup>12,13</sup> (Fig. 2). It can be seen that human  $\beta_2$ -microglobulin exhibited an electrophoretic mobility indistinguishable from that of the mouse counterpart. On reduction and alkylation both proteins displayed a retarded mobility compared with the untreated material, signifying that the mouse protein may contain a disulphide loop similar to that of human  $\beta_2$ -microglobulin<sup>14,15</sup>.

Further evidence for the great similarity between  $\beta_2$ -microglobulin and the small H-2 alloantigen polypeptide chain was obtained at primary structure level by peptide mapping experiments. Although chymotryptic as well as tryptic digests revealed peptides that were unique for the mouse protein, the majority of the peptides seemed to be shared between the two proteins. These data favour the hypothesis that the small H-2 alloantigen is the mouse homologue of human  $\beta_2$ -microglobulin.

The linkage of the mouse homologue of  $\beta_2$ -microglobulin to the larger H-2 alloantigen polypeptide chain may be a fortuitous result of the solubilisation procedure. Attempts were therefore made to establish a connection between the two polypeptides on the cell surface. It could thus be demonstrated that antibodies against human  $\beta_2$ -micro-





**Fig. 2** Urea-starch electrophoresis at pH 3.0 according to a modification<sup>13</sup> of the procedure outlined by Edelman and Poulik<sup>12</sup>. *a*, Untreated samples of the small H-2 alloantigen polypeptide chain labelled with <sup>125</sup>I and human  $\beta_2$ -microglobulin labelled with <sup>131</sup>I. *b*, The same material reduced with 0.1 M mercaptoethanol in 8 M urea for 30 min and alkylated with 0.12 M iodoacetic acid in the dark for 30 min. The radioactive polypeptides were isolated from the labelled antigens by gel chromatography in 3 M KCl. The electrophoresis was run for 2 h at a voltage of 22 V cm<sup>-1</sup>. After completion of the electrophoresis the gels were divided into segments of 2 mm width and assayed for radioactivity in a  $\gamma$ -well type scintillation counter. ●—●, <sup>125</sup>I; ○—○, <sup>131</sup>I.

globulin lysed more than 85% of mouse spleen cells in the presence of complement<sup>7</sup>. Fab'-fragments<sup>14</sup> against human  $\beta_2$ -microglobulin could completely abolish the cytotoxic effect of both H-2K<sup>a</sup> and H-2D<sup>a</sup> alloantisera (compare with ref. 7). Also, antibodies against human  $\beta_2$ -microglobulin cocapped FITC-labelled antibodies directed against H-2K<sup>a</sup> or H-2D<sup>a</sup> (ref. 6). It is thus conceivable that the mouse  $\beta_2$ -microglobulin counterpart is a true subunit of the H-2 alloantigens coded for both by the K and D ends of the major histocompatibility complex not only when liberated from the cell membrane but also on the intact cell surface.

We thank Dr H. Wigzell for supplying the alloantisera used in this study. The technical assistance of Miss Yvonne Fernstedt is acknowledged. Financial support was obtained from the Swedish Cancer Society and the Swedish Medical Research Council.

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Received December 28, 1973; revised March 15, 1974.

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## Induction of lymphokine production by EAC and of blastogenesis by soluble mitogens during human B-cell activation

Two groups of human lymphocytes are distinguished on the basis of immune effector functions, membrane markers and *in vitro* mitogen reactivity. Thymus-derived (T) lymphocytes mediate cellular immunity either directly or by secreting pharmacologically active lymphokines<sup>1</sup>, and are implicated in facilitating antibody responses<sup>2</sup>. In contrast, bone marrow-derived (B) lymphocytes are thought to produce only antibodies. Studies in the guinea pig, however, indicate that, appropriately stimulated, B cells can produce macrophage migration inhibitory factor (MIF)<sup>3</sup>. We have now found that the erythrocyte-antibody (19S)-complement (EAC) rosetting method, used to obtain pure human peripheral blood B cells, activates these B cells to produce mononuclear cell chemotactic (CTX) and mitogenic (MF) lymphokines. Furthermore, we have investigated the mechanism of B cell activation by EAC and the effects of mitogenic lymphokines on human T and B lymphocytes.

T and B lymphocytes were obtained as described in the legend to Table 1. Between 45 and 75% of peripheral blood lymphocytes were recovered as E-rosette-forming cells and 5-35% as EAC-rosette-forming cells. These high rates of recovery suggest that the subsequent biological studies are representative of T and B cell functions.

Purity of T and B cells was assessed by immunofluorescent staining of membrane-associated immunoglobulins, a characteristic of B cells. Different anti-human immunoglobulin sera were used: two conjugated with fluorescein (C20F-341, Meloy Laboratories), and 3031612 (Baltimore Biological Laboratories, Cockeysville, Maryland) and the other with rhodamine (6481, Cappel Laboratories, Dowingtown, Pennsylvania). All sera were titred to find their optimum staining concentrations. Rosetted cells ( $1.0 \times 10^6$ ) were treated with isotonic ammonium chloride lysing buffer, washed and kept at 3° C. A volume of 0.1 ml conjugated serum was added and the pelleted cells were incubated for 30 min at 4° C, washed three times in chilled azide-BSA buffer and wet mounts were made. In four experiments, the mean purity of T cells was  $95\% \pm 1$  and of B cells,  $97\% \pm 2$  with  $< 0.5\%$  monocyte contamination in either population.

**Table 1** Blastogenic responses of human T and B lymphocytes

|                        | T cells |                | B-EAC |             | Dissociated B cells |               |
|------------------------|---------|----------------|-------|-------------|---------------------|---------------|
|                        | N       | mean           | N     | mean        | N                   | mean          |
| Control                | (19)    | 87 ± 13        | (15)  | 157 ± 33    | (4)                 | 135 ± 12      |
| PHA (1 µg per well)    | (19)    | 16,404 ± 2,934 | (17)  | 4,926 ± 981 | (4)                 | 5,513 ± 3,404 |
| Con A (10 µg per well) | (14)    | 9,687 ± 2,471  | (12)  | 1,780 ± 685 | (3)                 | 4,002 ± 2,282 |

T and B cells (93–99% purity) were prepared from FH-separated (30–200ml) heparinised peripheral blood by a modification of a sequential rosetting technique unpublished results of W. West, J. McCoy, T. Doering and R. Herberman. Washed mononuclear cells were sequentially rosetted at 30° C with sheep erythrocytes (E) and foetal calf sera (heat-inactivated E absorbed). T cells were separated from non-rosetting cells on two consecutive Ficou-Hypaque (FH) gradients. NRFC were then rosetted with E coated with (19S) anti-E antibody-mouse complement (EAC) at 37° C and EAC-rosette-forming cells separated on two consecutive FH gradients. B-EAC-rosettes were dissociated with rabbit anti-C3 globulin (1 mg IgC per  $1 \times 10^7$  cells; Cappel Laboratories) at 37° C for 30 min. Dissociated B cells were separated from EAC on FH gradients, washed and diluted to  $2 \times 10^6$  viable cells per ml in RPMI-1640 (NIH) supplemented with a final concentration of 0.5% human AB sera and aliquoted in 0.1 ml volumes into microtitre trays. B and T cells with their adherent rosettes cells were diluted and aliquoted in a similar manner. The test mitogens PHA or con A were added in 0.1 ml volumes to triplicate wells and trays cultured for 72–96 h in 5% CO<sub>2</sub>–95% air at 37° C; 18 h before collection 3.0 µCi of <sup>3</sup>H-TdR (6 Ci mol<sup>-1</sup>, Schwarz/Mann) was added to each well. Cultures were processed automatically and total radioactivity incorporated into DNA was assessed. N represents the number of independent experiments used to calculate the results. Data are expressed as the mean c.p.m. of triplicate cultures ± s.e.

T and B cells with adherent E or EAC rosettes were placed in culture (as described in the Table legends) and stimulated with medium, concanavalin A (con A) or phytohaemagglutinin (PHA). Levels of incorporation of tritiated thymidine (<sup>3</sup>H-TdR) in control unstimulated cultures in both populations were quite low (Table 1); with a mean of 87 c.p.m. ± 13 for T cells and 157 ± 33 for B cells. Mitogen-stimulated T cells incorporated considerable <sup>3</sup>H-TdR and to a lesser degree B cells were also stimulated by soluble PHA and con A.

We investigated whether B cell responses to mitogens were due to PHA and con A adsorbed on the EAC, since particulate mitogens stimulate B cells<sup>4</sup>. B cells were dissociated from erythrocytes with anti-C3 antibody<sup>5</sup> and separated by Ficoll-Hypaque (FH) gradient centrifugation. Dissociated B cells were viable as assessed by trypan blue exclusion and still gave PHA and con A-induced proliferative responses equivalent to B-EAC cell cultures. Even transient EAC binding to C3 receptors may have lowered the threshold for B cell stimulation so that the subsequent interaction with soluble mitogens induced blastogenesis.

We then investigated whether purified T and B cells can be activated by soluble mitogens to make mediators as well as to proliferate. Supernatants from medium, PHA and con A-stimulated T and B cell cultures were assessed for CTX activity as before<sup>6</sup>. Table 2 shows that supernatants from T cell cultures which had been activated with PHA or con A to synthesise DNA also had appreciable CTX activity (30–60 cells per 20 fields), while unstimulated control supernatants, reconstituted after culture with mitogen, had essentially none (8–9 cells per 20 fields). The mean values of the reconstituted controls were equivalent to those obtained with medium. In contrast, all B cell supernatants contained equal levels of CTX activity irrespective of whether they were from unstimulated, reconstituted or mitogen-stimulated cultures. The generation of CTX activity in B cell cultures did not correlate with <sup>3</sup>H-TdR incorporation (Table 1). The CTX activity in both control and mitogen stimulated B-EAC cell supernatants was two to five times greater than in supernatants from mitogen-stimulated T cell cultures, suggesting that CTX activity in B-EAC cell culture supernatants was not derived from the few contaminating nonimmunoglobulin-bearing cells. Furthermore, mitogen-stimulated B-EAC cultures did not contain more CTX than supernatants from control cultures. This indicates that the contaminating cells were not producing lymphokines, as would be expected if they were T cells; this is another indication of the purity of our B cell preparations.

To assess whether the attachment through the C3 receptor non specifically induced the production of lymphokines, B-EAC cell rosettes were dissociated with

rabbit anti-mouse C3 globulin<sup>5</sup> (as described in Table legends) and dissociated B cells were cultured with or without mitogen. Supernatants from unstimulated B cells dissociated from EAC were devoid of high CTX activity compared with supernatants from cultures of undissociated B-EAC cell rosettes (Table 2). These data suggests that the attachment of EAC to B cells stimulates lymphokine production. But EAC binding was either insufficient to cause concomitant B cell blastogenesis or blastogenesis requires a different signal. In control experiments, E and EA (19S antibody) which do not bind to B cells failed to stimulate either lymphokine production or blastogenesis; however, EA (7S antibody) which binds to B cell membrane Fc receptors also induced B cell lymphokine production (B. F. M., L. C. A., and J. Jardszewski, unpublished data). Therefore, the induction of B cell lymphokine production seems to be facilitated by agents which bind to specific B cell membrane receptors. The biological activity of B cells dissociated from their adherent EAC was further assessed in terms of their ability to produce lymphokines after soluble mitogen stimulation (Table 2). Con A and PHA stimulation of dissociated B cell cultures induced CTX supernatant activity comparable with that of B-EAC cultures and twice as great as those of mitogen-stimulated T cells. The dissociated B cell culture supernatant levels of CTX were too high to be attributed to the low percentage of contaminating immunoglobulin-negative cells; however, we cannot ignore the possible contribution of helper cell effects. Table 2 suggests that with appropriate stimulation both human T and B lymphocytes produce lymphokines. Further, lymphokine was produced in B-EAC cultures in the absence of concomitant blastogenesis; an observation consistent with the reported dichotomy between lymphocyte proliferation and macrophage inhibitory factor synthesis<sup>7,8</sup>.

CTX activity in B cell supernatants was not derived from the two chemotactic complement peptides, C3a and C5a. First, B cells purified using EAC prepared with C5 deficient mouse sera still generated CTX activity equivalent to B cells separated with EAC prepared with normal mouse sera and second, the treatment of B cell supernatants with antisera to C3 and C5 did not appreciably decrease their chemotactic activity; specificity of antisera was assayed by immunodiffusion. The CTX activity found in B cell culture supernatants was therefore presumed to be produced by B cells and seems to have physicochemical characteristics similar to those produced by T cells (L. C. A., B. F. M., and B. Chassy, in preparation).

Supernatants from T and B cell cultures were also assessed for MF. We asked two questions: first, could purified T and B cells produce MF and second, could B, as well as T cell MF stimulate pure T or B cells (Table 3).



**Table 2** Chemotactic lymphokine activity in T and B lymphocyte culture supernatants

|                                 | N   | T cell supernatants |            |     | E/C | N      | B-EAC cell supernatants |            |     | E/C  | N      | Dissociated B cell supernatants |            |     | E/C |
|---------------------------------|-----|---------------------|------------|-----|-----|--------|-------------------------|------------|-----|------|--------|---------------------------------|------------|-----|-----|
|                                 |     | reconstituted       | stimulated |     |     |        | reconstituted           | stimulated |     |      |        | reconstituted                   | stimulated |     |     |
| Medium                          | (5) | —                   | 9±2        | —   | (6) | —      | 93±7                    | —          | (6) | —    | (6)    | —                               | 22±6       | —   | —   |
| PHA (µg ml <sup>-1</sup> )      | (9) | 9±2                 | 66±3       | 7.3 | (4) | 82±2   | 99±14                   | 1.2        | (2) | 18±3 | 104±11 | 18±3                            | 114±11     | 6.3 | 5.7 |
| Con A (10 µg ml <sup>-1</sup> ) | (7) | 8±1                 | 30±2       | 3.8 | (2) | 134±12 | 142±10                  | 1.1        | (2) | 18±3 | 114±11 | 18±3                            | 114±11     | 6.3 | 5.7 |

T and B lymphocytes at  $4 \times 10^6$  per 2 ml culture (RPMI-1640; 0.5% human AB sera supplemented) with or without stimulant were incubated at 37° C in 5% CO<sub>2</sub>-95% air for 24 h. B-EAC rosettes immediately after purification were treated with rabbit anti-mouse C3 globulin (1 mg IgG per  $1 \times 10^7$  cells; 37° C for 30 min with constant shaking). Dissociated B cells were separated from EAC and undissociated B cells by two FH gradient centrifugations. Total viable dissociated B cells were counted by trypan blue dye exclusion, then cells were diluted and cultured as described. Supernatants were collected and tested for CTX activity. The test population of mononuclear cells (MNC) used in the chemotactic assays was separated from human peripheral blood on FH gradients, washed twice in Gey's balanced salt solution (pH 7.0) and diluted to  $5 \times 10^6$  cells ml<sup>-1</sup>. Target MNC and culture supernatants were placed in modified Boyden chambers separated by a 5 µm polycarbonate filter (Nuclepore, Wallabs Inc.). The chambers were incubated for 90 min at 37° C in air<sup>8</sup>; the filters were removed, stained, and the cell migration was quantitated. Results are expressed as the mean number of migrating cells per 20 oil fields  $\pm$  s.e. of triplicate samples. N represents the number of experiments used to calculate the mean. The E/C values represent the ratios of the CTX activity in supernatants from stimulated cultures divided by the activity in their reconstituted controls.

Supernatants from con A-stimulated and reconstituted T cell cultures were passed through Sephadex G-100 to remove most added con A (ref. 9); supernatants reconstituted with con A after incubation served as controls for the mitogenic activity of any residual con A. Since there was no difference in results for autologous and allogeneic secondary cultures the data were combined. Supernatants from con A-stimulated T cell cultures could activate both T and B cells. Reconstituted T cell supernatants had slightly more mitogenic activity than control supernatants, probably due to residual con A. Supernatants from control EAC-B cells

CTX activity ( $144 \pm 5$  cells per 20 fields) irrespective of the presence of T cells; control unrosetted and unstimulated mononuclear cell supernatants had no activity. Second, the addition of T cells to purified B-EAC rosette cultures in ratios of 1:10 and 1:1 neither suppressed nor increased the generation of B cell CTX activity.

We conclude first that human peripheral blood T and B lymphocytes proliferate in response to soluble mitogens previously thought to stimulate only T cells. One explanation for this is a helper effect by the few contaminating immunoglobulin negative cells. Alternatively the inter-

**Table 3** Mitogenic lymphokine activity in supernatants of human T and B lymphocyte cultures

| Primary cultures |             |               |      | Secondary target cell cultures |      |      |                    |
|------------------|-------------|---------------|------|--------------------------------|------|------|--------------------|
| cell type        | stimulant   | reconstituted | (N)  | T lymphocytes mean             | E/C  | N    | B lymphocytes mean |
| —                | —           | Medium        | (3)  | 70±13                          | 1.0  | (4)  | 59±16              |
| T                | Medium      | —             | (3)  | 128±19                         | 1.8  | (4)  | 49±8               |
| T                | Medium      | Con A         | (5)  | 444±63                         | 6.3  | (3)  | 110±80             |
| T                | Con A       | —             | (5)  | 4,690±460                      | 70.6 | (3)  | 1,406±698          |
| —                | —           | Medium        | (15) | 87±35                          | 1.0  | (18) | 146±108            |
| B                | EAC Binding | —             | (15) | 429±97                         | 4.9  | (18) | 579±107            |

T and B lymphocyte culture supernatants were prepared as described for Table 2. In experiments using con A as a stimulant, control supernatants were reconstituted after incubation with an equivalent concentration of con A (10 µg ml<sup>-1</sup>) and reconstituted and stimulated (experimental) supernatants (4–6 ml) both passed through 10 ml Sephadex G-100 columns<sup>9</sup> (binding capacities of 300 µg con A per ml of wet G-100). Rosetted T and B target cells were prepared as previously described from allogeneic and autologous donors. A microtray system was used to assess MF activity; 0.1 ml containing  $2 \times 10^5$  viable cells and 0.1 ml or 0.05 ml of each test supernatant or RPMI-1640 added in to each well; assays were performed in triplicate wells for each variable. Trays incubated at 37° C in 5% CO<sub>2</sub>-95% air for 72 h; 18 h before collection 3 µCi <sup>3</sup>H-TdR was added to each well. Cultures were processed automatically and the total radioactivity incorporated into DNA assessed. N represents the number of supernatant pairs assessed with the results expressed as the mean (c.p.m.)  $\pm$  s.e. The E/C values represent the stimulation index.

to which no mitogen was added and which did not incorporate <sup>3</sup>H-TdR also stimulated both T and B cell blastogenesis in autologous and allogeneic secondary cultures. This MF activity was not ascribed to conditioned medium effects since control T cell supernatants had no stimulatory effects. Significantly more blastogenesis occurred in EAC-B-cell-supernatant-stimulated secondary cultures than in secondary cultures to which only medium was added; however, blastogenesis did not approach that produced by con A-stimulated T cell supernatants. Thus, human B cells activated by EAC-rosetting produced MF similar to the MF in supernatants of con A-stimulated T cell cultures which activated both T and B cells. Furthermore, the incubation of purified T cells with EAC induced neither blastogenesis nor MF production indicating that EAC binding to B cells was the cause of MF synthesis.

Further control experiments were performed to assess whether lymphokine production by human B cells resulted from removal of suppressor T cells. First, B-EAC rosettes were formed but not separated from the total peripheral blood mononuclear cell population, and cultured for 24 h. Supernatants from these cultures contained high levels of

action of activated EAC-3 with B cells through their C3 receptors, may have provided an initial signal so that subsequent binding of soluble PHA or con A became sufficient to stimulate B cell blastogenesis<sup>10</sup>. These findings are consistent with reports that human B cells purified on immunoabsorbent columns also respond to soluble mitogens<sup>11,12</sup>. These immunoabsorbents, like EAC rosettes, may lower the threshold for B cell activation by binding to a membrane receptor.

Second, the binding of EAC and EA (7S) to human B lymphocytes induced CTX and MF synthesis but not concomitant blastogenesis. In contrast, PHA and con A-stimulated dissociated B cells to both proliferate and synthesise lymphokines. These data indicate that lymphokine synthesis and blastogenesis are either functions of different lymphocyte populations or require different degrees of membrane stimulation for their initiation. Finally, the demonstration that B lymphocytes produce a mitogenic lymphokine which effects T cells suggests a previously unrecognised mechanism of immune regulation.

This work was supported by the National Institute of Dental Research to Litton Bionetics Inc.

*Note added in proof:* The report<sup>14</sup> that purified human B cells fail to proliferate in response to soluble T cell mitogens conflicts with our and other findings<sup>11,12</sup>.

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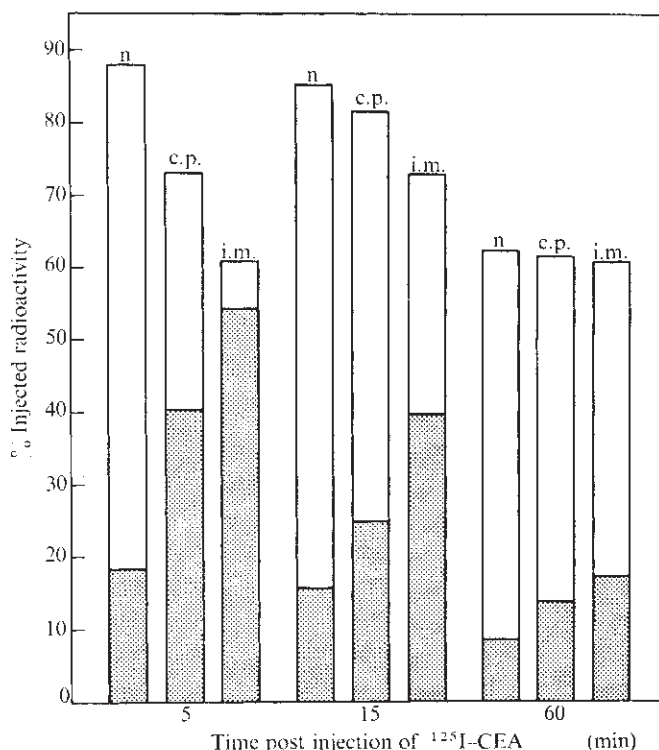
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## Altered metabolism of carcinoembryonic antigen in hamsters bearing GW-39 tumours

THE liver is well known for its role in the catabolism of a variety of plasma glycoproteins<sup>1</sup>. Liver-mediated clearance of a tumour glycoprotein antigen, the carcinoembryonic antigen (CEA) associated with human gastrointestinal adenocarcinomas<sup>2</sup>, has been demonstrated in dogs and rabbits<sup>3</sup>. Since circulating CEA is being extensively investigated for its diagnostic value in the detection of cancer patients, we were prompted to determine the metabolism of CEA in a xenogeneic species which supports the growth of a CEA-producing colonic cancer.

Female Syrian hamsters, 110–180 g, were heterografted with a CEA-producing colonic adenocarcinoma of human origin continuously propagated in hamsters<sup>4</sup>. CEA, purified from liver metastases of human colonic adenocarcinomas (E. S. Newman, H. J. Hager and H. J. H., manuscript in preparation), was radiolabelled with <sup>125</sup>I by the chloramine-T method<sup>5</sup>. Animals were injected intracardially with 7–9  $\mu$ Ci of aggregate-free <sup>125</sup>I-CEA (40–80  $\mu$ Ci  $\mu$ g<sup>-1</sup>) diluted in phosphate buffered saline containing 10% autologous serum. At timed intervals, each animal was exsanguinated by cardiac puncture, its organs were removed and radioactivity was determined in each and in 1 ml of plasma, using a Model 3375 Packard gamma scintillation counter. The radioactive material in the plasma, urine, and liver was characterised by Sepharose 6B column chromatography and by immunoreactivity with goat anti-CEA antibody in the radioimmunoassay developed by Hansen *et al.*<sup>6</sup>.

Figure 1 shows that in normal hamsters, 80–90% of the



**Fig. 1** Clearance of <sup>125</sup>I-CEA in normal and GW-39 tumour-bearing hamsters. Shaded area of each bar is the percentage of radioactivity remaining in the plasma of normal hamsters (n), and hamsters with cheek pouch (c.p.) or intramuscular (i.m.) tumours. Clear area of bar is the percent of radioactivity accumulated in the liver. The c.p. and i.m. tumours ranged in size from 2–4 and 20–35 g, respectively.

injected radioactivity was cleared by the liver within 5 min. This level of radioactivity was maintained in the liver until 30 min, at which time it began to decrease. Initially, 10–20% of the injected radioactivity was not cleared from the circulation; at 12 h, less than 5% was recovered from either the liver or blood. The accumulation of radioactivity in the urine coincided with its disappearance from the liver and by 24 h, 95% of the injected dose of radioactivity was found in the urine. At all times, the distribution of radioactivity was less than 1.0% in the spleen, kidneys, lungs, heart, and faeces, whereas 10–30% localised in the intestinal tract. The radioactivity reached a peak of 20–35% within the small bowel at 3 h and then declined, while a maximum of 10–15% was recovered in the large bowel 3 h later.

When radiolabelled CEA was injected into hamsters bearing cheek pouch and intramuscular GW-39 tumours, a decrease in the clearance of plasma radioactivity was observed in hamsters bearing tumours at either site, being more marked in animals with intramuscular tumours (Fig. 1). Initially, the increase in vascular radioactivity corresponded to a decrease in the quantity present in the liver. But as the plasma radioactivity decreased, its level began to rise in the liver, and by 60 min, the radioactivity in both the plasma and liver of tumour-bearing hamsters was similar to that of normal animals. The distribution of radioactivity among the other organs studied in tumour hamsters was identical to that found in normal hamsters.

The radioactivity which was present in a 30 min and 3 h liver sample of a normal hamster was characterised on Sepharose 6B columns calibrated with the preinjection radiolabelled CEA (Fig. 2). The first radioactive peak in a 30 min liver sample cochromatographed with the pre-injection antigen, whereas at 3 h, this peak eluted at a slightly lower molecular weight. The second peak in both cases appeared after the total column volume was voided

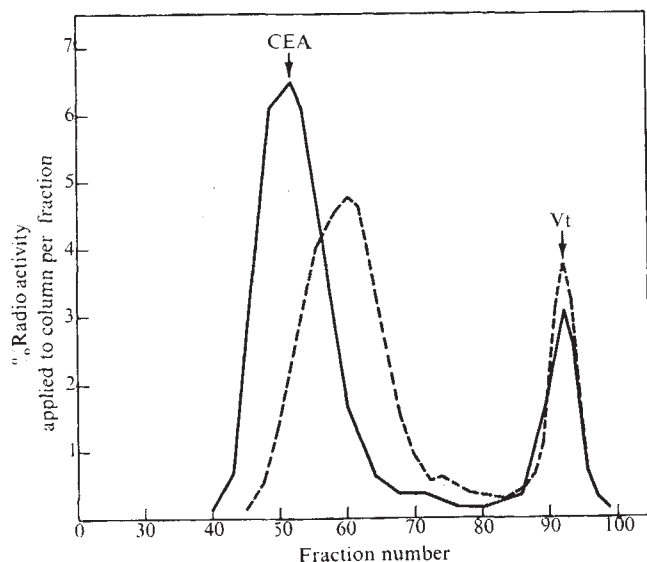


and represented low molecular weight materials which lacked immunoreactivity with goat anti-CEA antibody. The percentage of this material in the liver was 10–15% from 30 to 360 min. Following perchloric acid (PCA) extractions, the radioactivity sequestered by the liver during the latter times was 80–90% immunoreactive.

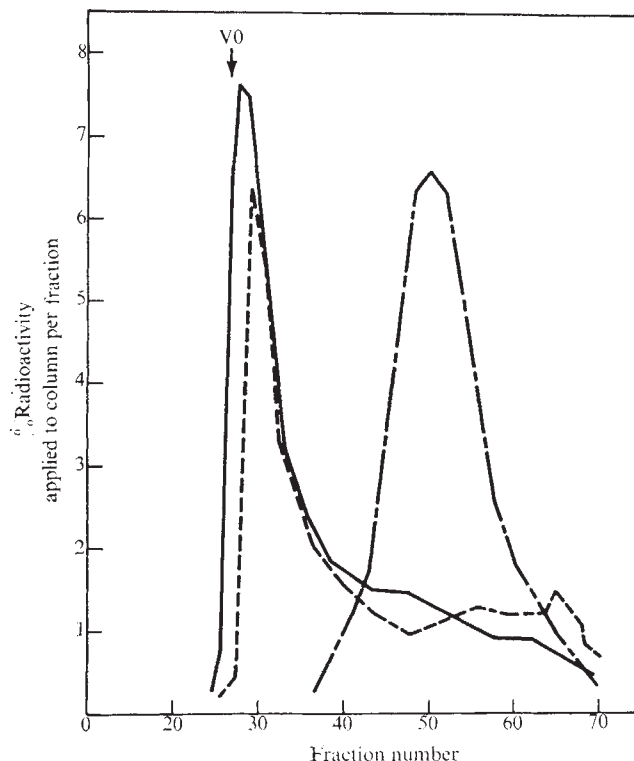
The radioactivity not initially cleared by the liver and remaining in the plasma at 5 min was identical, according to Sepharose 6B chromatography, to the preinjection CEA. Subsequently, a low molecular weight, nonimmunoreactive radioactive peak, similar to that in the liver, gradually appeared and accompanied a decrease in the quantity of radioactive material which eluted as the preinjection CEA. By 3 h, the relative percentages of the former and latter were 70% and 25% of the plasma radioactivity, respectively. At 5 min, more than 70% of the plasma radioactivity was immunoreactive before PCA extraction but decreased to 10% by 3 h, owing to the raised levels of the low molecular weight radioactive material. Loss of the latter material during dialysis of 30 min to 3 h plasma samples extracted with PCA yielded material which was 70–75% immunoreactive. All of the radioactivity which accumulated in the urine at 30 min or 24 h eluted as the low molecular weight, nonimmunoreactive material.

Figure 3 shows that a major portion of the radioactivity in both 15 and 60 min plasma from hamsters bearing intramuscular tumours appeared in the void volume after Sepharose 6B chromatography. Although there was a 20% drop in the blood's radioactivity during this time, the relative percentage (45%) of the void volume material remained approximately the same.

Our results with normal hamsters are in general agreement with the report by Shuster *et al.*, in which the metabolism of CEA was investigated in dogs and rabbits<sup>3</sup>. Slight differences in the initial plasma clearance rates are most likely a result of variations in the sialic acid content of the CEA preparations used in the different studies, as previously reported for other serum glycoproteins<sup>7</sup>. We have demonstrated a delay in the blood plasma clearance of radiolabelled CEA in GW-39 tumour-bearing hamsters.



**Fig. 2** Chromatography of 30 min (—) and 3 h (---) liver radioactivity on Sepharose 6B columns. Livers were homogenised in distilled water, 1:4 (w/v), and centrifuged at 12,000g for 30 min. After applying a sample from the supernatant to a 2.6 × 90 cm Sepharose 6B column, 5 ml fractions were collected and assayed for radioactivity. 85 to 98% of the radioactivity applied was recovered. Arrows, total column volume (Vt), and the elution volume of the preinjection radiolabelled CEA.



**Fig. 3** Chromatography of normal and GW-39 tumour-bearing hamster plasma radioactivity on Sepharose 6B. 85 to 95% of the radioactivity applied was recovered. Arrow, void volume (V0); (---), 15 min plasma sample from hamster with tumour; (---), 60 min plasma sample from hamster with tumour; (---), 15 min plasma sample from normal hamster.

Since the GW-39 tumour elicits the formation of a hamster anti-CEA IgM antibody<sup>8</sup>, the plasma radioactive macromolecule which appears in these animals is probably a CEA-antibody complex. Future studies will attempt to determine whether this antibody interferes with CEA clearance or is incidental to a more general state of tumour-induced suppression of liver function.

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## Regulation of the corticosteroid inducibility of tyrosine aminotransferase in interspecific hybrid cells

TYROSINE aminotransferase (TAT) is not inducible by corticosteroid hormones in heterokaryocytes and hybrid cells resulting from the fusion of rat hepatoma cells with mouse fibroblasts<sup>1,2</sup>. Fusion of rat hepatoma cells with human fibroblasts also results in the loss of TAT inducibility, but because of the unidirectional loss of human chromosomes in the resulting hybrid cells<sup>3</sup>, inducibility of TAT is re-expressed in some clones<sup>4</sup>. The suppression or modulation of the expression of TAT inducibility in such hybrid clones seems to be related to presence of the human X chromosome<sup>4</sup>. Since rat-human hybrid clones in which TAT inducibility is suppressed have the same corticosteroid receptor activity as the rat parental cells<sup>4</sup>, the absence of inducibility cannot be ascribed to a decrease in corticosteroid receptor activity.

To identify the biochemical event involved in the suppression of the TAT inducibility in interspecific hybrid clones, we have analysed a series of rat-mouse and rat-human suppressed hybrid clones for corticosteroid receptor activity and for their ability to transfer the dexamethasone-receptor complex to the nucleus. The rat hepatoma cells (Fu5AH) used are resistant to 15  $\mu\text{g ml}^{-1}$  of 8-azaguanine and deficient in hypoxanthine-phosphoribosyltransferase (HPRT) activity<sup>5</sup>. They were hybridised with mouse Cl-1D cells which are derived from L cells<sup>6</sup> and are resistant to 30  $\mu\text{g ml}^{-1}$  of BUdR, and with human KOP fibroblasts which are derived from a subject with a translocation of the long arm of the human X chromosome to D-14 [t(14q, Xq)] (ref. 7). Fu5AH cells were fused with either Cl-1D cells or KOP cells in the presence of  $\beta$ -propiolactone-inactivated Sendai virus in Eagle's minimal essential medium (MEM) at pH 8.0 (ref. 8). Immediately after fusion, hypoxanthine-aminopterin-thymidine<sup>9</sup> (HAT) selective medium was added to the cultures. After 14 d, colonies of mouse-rat hybrid cells were isolated. These cells were then cloned in microtest plates.

After fusion of human and rat cells the cultures were maintained and passaged in HAT medium to eliminate human fibroblasts. Colonies of hybrid cells were then obtained 4-6 weeks after fusion, permitted to grow and subsequently cloned.

The chromosomes of the parental rat, mouse and human cells and of cells of each hybrid clone were stained by a modification of Seabright's Giemsa banding method<sup>8,10</sup>, and a minimum of 20 karyotypes of each hybrid clone was analysed to identify the origin of the chromosomes of the hybrid cells. Chromosome preparations were treated with a solution of trypsin (0.05%) and EDTA (0.02%) (Gibco) for 5 min, rinsed with medium containing 5% foetal calf serum and then rinsed with Hanks solution. The slides were then stained in 2% Giemsa (pH 6.8) for 5 min, rinsed with pH 6.8 buffer (made by dissolving one tablet of G.T. Gurr pH 6.8 buffer in 100 ml water) and dried in humidified hot air.

Of the three parental cells studied, TAT was inducible by dexamethasone only in the rat Fu5AH cells (> four-fold induction) (Table 1).

In all the rat-mouse hybrid clones the TAT baseline activity was not expressed and the enzyme was not inducible by dexamethasone (specific activity = 0). In the rat-human (Fu5AH  $\times$  KOP) hybrid clone 7, in which the only human chromosome present was the t(14q, Xq), the baseline TAT activity was similar to that of the rat parental cells (Table 1) whereas the inducibility of TAT was significantly reduced in comparison with that of the rat parental cells.

The rat-mouse hybrid clones contained between 83 and 103 chromosomes which could be identified by Giemsa banding (Fig. 1, Table 1). In these clones, most of the chromosomes of the rat and mouse parental cells were retained (Fig. 1), but occasionally rat chromosomes were preferentially lost.

**Table 1** Induction of TAT in rat-mouse and rat-human hybrid cells

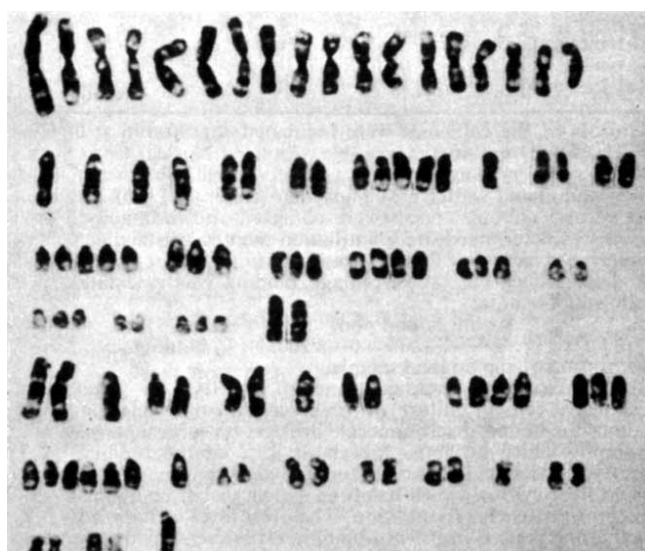
| Cell line       | Dexamethasone | TAT specific activity* | Average chromosome no. |
|-----------------|---------------|------------------------|------------------------|
| Parental        |               |                        |                        |
| Fu5AH           | —             | 0.5                    | 52                     |
|                 | +             | 2.2                    |                        |
| Cl-1D           | —             | 0                      | 52                     |
|                 | +             | 0                      |                        |
| KOP             | —             | 0                      | 46                     |
|                 | +             | 0                      |                        |
| Hybrids         |               |                        |                        |
| Rat-mouse Cl 5  | —             | 0                      | 99                     |
|                 | +             | 0                      |                        |
| Rat-mouse Cl 7  | —             | 0                      | 95                     |
|                 | +             | 0                      |                        |
| Rat-mouse Cl 8  | —             | 0                      | 101                    |
|                 | +             | 0                      |                        |
| Rat-mouse Cl 10 | —             | 0                      | 89                     |
|                 | +             | 0                      |                        |
| Rat-mouse Cl 16 | —             | 0                      | 91                     |
|                 | +             | 0                      |                        |
| Rat-mouse Cl 17 | —             | 0                      | 98                     |
|                 | +             | 0                      |                        |
| Rat-mouse Cl 18 | —             | 0                      | 103                    |
|                 | +             | 0                      |                        |
| Rat-mouse Cl 20 | —             | 0                      | 83                     |
|                 | +             | 0                      |                        |
| Rat-human Cl 7  | —             | 0.7                    | 79                     |
|                 | +             | 1.1                    |                        |

Almost confluent monolayers of parental and hybrid cells were kept in serum-free medium with or without 10  $\mu\text{M}$  dexamethasone for 18 h. The cells were collected after centrifugation at 800g for 10 min at 4° C, washed, and resuspended in extraction medium (0.15 M KCl, 1 mM EDTA, 0.1 mM potassium phosphate buffer at pH 7.6). This suspension was sonicated by two 10 s bursts with a chilled probe of Branson sonifier at a setting of 5 A. The sonicated suspension was immediately centrifuged in a RC-2 Sorvall centrifuge at 20,000g for 15 min at 4° C. The supernatant solutions were tested for TAT by the method of Canellakis and Cohen<sup>11</sup> and for proteins by the method of Lowry *et al.*<sup>12</sup>.

\* TAT specific activity =  $\mu\text{mol } p$ -hydroxyphenyl pyruvate formed per 10 min (37° C) per mg of protein.

Such chromosomal loss seemed to be limited and usually involved less than 40% of the rat chromosomes (Fig. 1).

To survive in HAT medium<sup>4</sup>, the rat-human hybrid cells had to retain the long arm of the human X chromosome which was translocated to D-14<sup>7</sup> (Fig. 2), since the human HPRT gene is located on the long arm of the X chromosome<sup>7</sup>.



**Fig. 1** Rat-mouse hybrid Cl 18. All the mouse Cl-1D chromosomes are present in this clone (upper four rows). Forty rat chromosomes are present in this hybrid (last three rows).



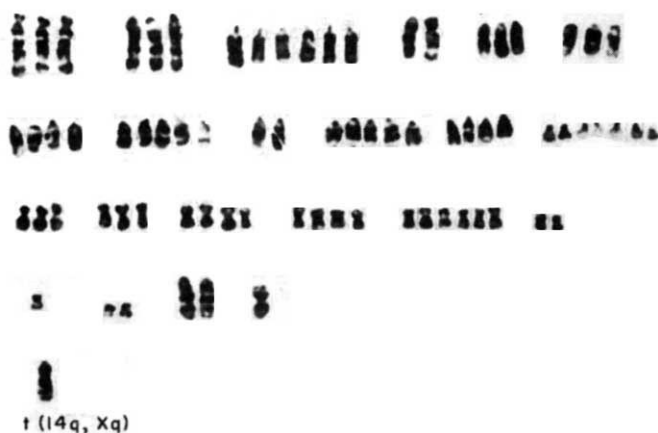


Fig. 2 Rat-human hybrid Cl 7. Seventy-five rat chromosomes are present in this hybrid. Only the human t (14q, Xq) chromosome is present in this clone.

As Table 2 shows, both rat and mouse parental cells displayed corticosteroid receptor activity. The amount of receptor activity was higher in the mouse parental cells (Table 2), in which TAT could not be induced by dexamethasone (Table 1). The KOP cells, as well as WI38 human fibroblasts<sup>4</sup>, had low amounts of receptor activity (Table 2). Two noninducible mouse-rat hybrid clones (Table 1) displayed corticosteroid receptor activity which was higher than that of the parental mouse and rat cells (Table 2). The rat-human hybrid clone 7, in which TAT was only slightly inducible by corticosteroids, displayed higher dexamethasone-receptor activity (Table 2) than the rat hepatoma parental cells.

As Table 3 shows, transfer of radioactive dexamethasone to the nucleus proceeded to the same extent in both rat Fu5AH and mouse Cl-1D cells. In the three rat-mouse hybrid clones, in which TAT was not inducible (Table 1), the percentage of

radioactive dexamethasone recovered in the nuclei at various times after exposure to the hormone was slightly higher than in the rat and mouse parental cells.

In the rat-human hybrid clone 7, the bound dexamethasone nuclear transfer proceeded to the same extent as in the Fu5AH parental cells (our unpublished results).

Since the rat-human hybrid cells that retained the human X (long arm)/D-14 chromosome had a much lower TAT inducibility than did the rat Fu5AH cells (Table 1), the gene(s) modulating TAT inducibility by corticosteroids seems to be located on the long arm of the human X chromosome. Previous studies excluded this role for the human D-14 chromosome<sup>4</sup>.

Our results indicate that noninducibility of TAT in rat-mouse or rat-human hybrid cells cannot be attributed to an absence or decrease of dexamethasone-receptor activity. In fact the rat-human hybrid clone 7 in which TAT was only

Table 3 Nuclear transfer of <sup>3</sup>H-dexamethasone in rat-mouse hybrids

| Cell line       | 2 h  | % Nuclear transfer<br>4 h | 6 h  | 18 h |
|-----------------|------|---------------------------|------|------|
| Fu5AH           | 17.8 | 25.9                      | 34.1 | 20.5 |
| Cl-1D           | 26.9 | 26.5                      | 23.4 | 28.1 |
| Rat-mouse Cl 5  | 33.6 | 28.1                      | 30.6 | 41.4 |
| Rat-mouse Cl 8  | ND** | 56.3                      | 42.1 | 48.6 |
| Rat-mouse Cl 10 | ND** | 43.1                      | 54.5 | 49.3 |

Almost confluent monolayers of parental and hybrid cells were exposed to  $3.3 \times 10^{-8}$  M 1, 2, 4-<sup>3</sup>H-(N)-dexamethasone (specific activity 21.2 Ci mmol<sup>-1</sup>) in serum-free medium. At different intervals the cells were trypsinised, centrifuged and washed three times in ice cold phosphate-buffered saline (PBS). The cells were resuspended in hypotonic reticulocyte standard buffer (RSB), composed of 10 mM NaCl, 10 mM Tris-HCl, pH 7.4, and 1.5 mM MgCl<sub>2</sub>, and homogenised. The cytoplasmic fraction was centrifuged in a RC-2 Sorvall centrifuge at 20,000g for 15 min at 4°C. The nuclei were stripped of the outer nuclear membrane by double detergent procedure according to the method described by Penman<sup>16</sup>. The percentage of nuclear transfer of the dexamethasone-receptor complex was determined by the following formula:

$$\% \text{ Nuclear transfer} = \frac{\frac{\text{c.p.m.}}{\text{mg of nuclear proteins}}}{\frac{\text{c.p.m.}}{\text{mg of nuclear proteins}} + \frac{\text{c.p.m.}}{\text{mg of cytosol proteins}}} \times 100$$

We conclude that the activity of the hormone receptor has indeed been measured by our techniques for the reasons set forth in Table 2. Furthermore, nuclear translocation experiments ascertain the presence and biological activity of the receptor as shown by the results described in this table.

slightly inducible by dexamethasone had dexamethasone-receptor activity higher than that of the rat parental cells. In addition the mouse-rat hybrid cells have more dexamethasone-receptor activity than either of the parental cells. It is thus possible that in this case fusion of two cell types, both with dexamethasone-receptor activity, results in the formation of a hybrid cell with combined receptor activity of the two parental cells.

In all the clones tested in which TAT was not inducible but which had dexamethasone receptor activity, the transfer of radioactive dexamethasone to the nucleus proceeded to the same or greater extent than in the rat or mouse parental cells, indicating that the biochemical event which is missing in these noninducible hybrid clones is not the transfer to the nucleus of the dexamethasone-receptor complex. Therefore, the regulation of the inducibility of TAT in interspecific hybrid cells must take place either at the level of the binding of the dexamethasone receptor complex to the chromatin or at the transcriptional or post-transcriptional level. These problems are now being investigated.

We thank Irene Kieba, Nancy Shapiro and C. A. Wishman for technical assistance. This work was supported in part by grants from the US Public Health Services Division of

Table 2 Corticosteroid receptor activity of parental and hybrid cell lines

| Cell line       | Receptor activity<br>(% hormone binding) | TAT<br>inducibility |
|-----------------|------------------------------------------|---------------------|
| Fu5AH           | 21                                       | +++ +               |
| Cl-1D           | 45                                       | —                   |
| KOP             | 4                                        | —                   |
| Rat-mouse Cl 5  | 71                                       | —                   |
| Rat-mouse Cl 18 | 67                                       | —                   |
| Rat-human Cl 7  | 32                                       | +                   |

Cytosols of the cell lines were incubated for 90 min at 0°C with 2 nM 1,2,4-<sup>3</sup>H-dexamethasone (New England Nuclear Corp.). A load sample of 0.5 ml was added on a 10 ml syringe column of Sephadex G-25 and eluted with 1 mM phosphate buffer (pH 7.6). Pre-bound, bound and unbound pools were collected and radioactivity of the pools was determined in a scintillation mixture containing Triton-X, toluene and methanol fluor<sup>13</sup>. These experiments were done essentially as described earlier<sup>13</sup>. Percentage binding was calculated by the following formula:

$$\frac{\text{c.p.m. bound pool}}{\text{c.p.m. load sample}} \times 100 = \% \text{ binding}$$

Using radioactive dexamethasone at this level in glucocorticoid responsive cells in culture we observed that only one major dexamethasone-bound macromolecule appears on ion-exchange chromatography which accounts for about 95% of the binding<sup>14</sup>. This fraction is identical to the binder II dexamethasone binding protein of rat liver cytosol which has been shown to be the major hormone receptor protein<sup>15</sup>. In addition, when this level of radioactive dexamethasone is used in *in vitro* binding experiments with adrenalectomised liver cytosol, there is only one major peak on ion-exchange chromatography which is characterised as binder II, the hormone receptor (unpublished experiments). Thus, the values reported for binding of <sup>3</sup>H-dexamethasone in cytosol represent about 95% hormone receptor.

Research Resources, the National Cancer Institute and the National Institute of Arthritis, Metabolic and Digestive Diseases.

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## New biological activity following intravascular activation of the complement cascade

ACTIVATION of the complement cascade ( $C_1$ - $C_9$ ) by the classic or alternate pathways is associated with the development of several biological activities<sup>1</sup>. These include the elaboration of chemotactic factors ( $C_{3a}$ ,  $C_{5a}$ , and a trimolecular complex  $C_{5b,6,7}$ ), opsonic activity associated with the formation of  $C_{3b}$  from the third component, anaphylatoxin activity associated with the formation of  $C_{3a}$  and  $C_{5a}$  from the third ( $C_3$ ) and fifth ( $C_5$ ) components, and lysis of certain cells after the formation of lesions at the cell surface associated with fixation of the eighth ( $C_8$ ) and ninth ( $C_9$ ) components. We have detected another activity which follows intravascular activation of the complement cascade with the elaboration of the low molecular weight factor that produces an immediate neutropenia followed by marked neutrophilia.

Activation of the complement cascade intravascularly by the alternate pathway was achieved in New Zealand White rabbits or Hyline Sandy Lop rabbits, weighing approximately 2.5 kg, by intravenous injection 0.6-1.0 ml of purified cobra venom factor or 50 mg of a suspension of inulin (Sigma). The CVF was purified from the crude venom of *Naja naja* (Sigma) on DEAE cellulose and Sephadex G-200 columns after the method of Ballou and Cochrane<sup>2</sup>. The

complement activators were injected directly into the marginal ear vein of the rabbit and sequential heparinised venous blood samples were obtained. Leukocyte counts and Wright-stained differential counts were performed by standard techniques.

Figure 1 shows the effects of intravenous administration of the two activators of complement on neutrophils. A profound neutropenia occurred within 60-120 s and this was followed by neutrophilia within 0-3 h. Figure 2 shows the time course of depletion of the opsonin activity and antigenic levels of  $C_3$  in rabbit serum after challenge with CVF. Opsonic activity was determined by counting the total number of zymosan particles ingested in 100 neutrophils (PMN). Values are expressed as percentages of a parallel control. The alterations in PMN counts in Fig. 2 coincided more closely with depletion of opsonic activity than with reduction in antigenic  $C_3$  levels.

To determine whether the biological activity was associated with complement activation, rabbits were first depleted of complement by sequential injections of purified CVF as described by Brown<sup>4</sup>. When the antigenic  $C_3$  level was less than 10% the animal was challenged with 1.0 ml of CVF. In the complement-depleted animal there was little effect on PMN counts after rechallenge with 1.0 ml of CVF.

Further substantiation that the biological effect was associated with complement was obtained by infusing 40 ml of fresh rabbit plasma into an animal that was complement-depleted but still had circulating CVF. It has been demonstrated that, after sequential intravascular administration of CVF, the CVF continues to circulate until antibodies against it are formed<sup>5</sup>. This infusion of the 40 ml of fresh heparinised plasma into a complement-depleted rabbit (opsonic index <10%) caused acute neutropenia followed soon by neutrophilia (Fig. 3).

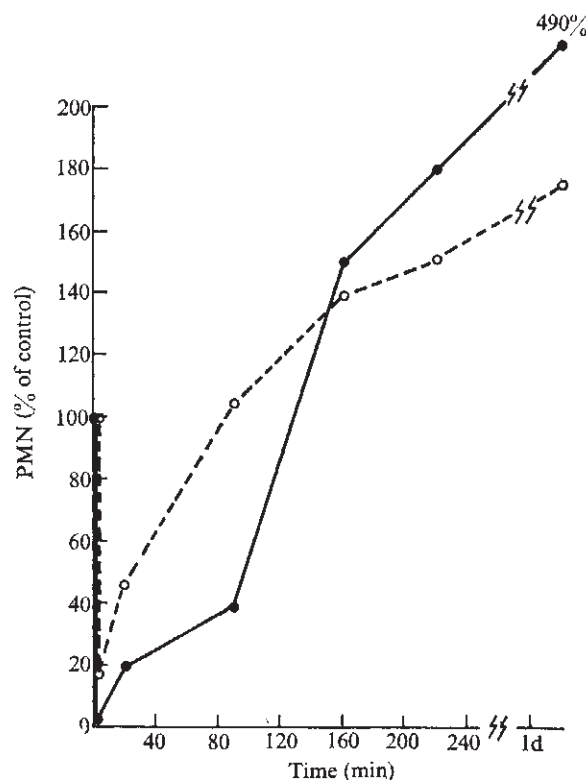


Fig. 1 Effects of intravenous administration of 1.0 ml of CVF (●) or 50 mg of inulin (○) on rabbit peripheral blood PMN counts. Values are expressed as percentages of control value, which was obtained 1-5 min before infection. The values are representative experiments, selected from more than five individual observations, all done in duplicate.



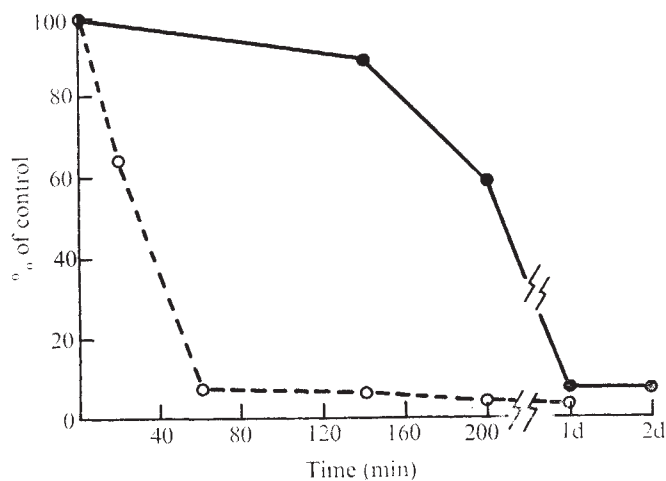
Both CVF and inulin activate complement by the alternate pathway which results in formation of a  $C_3$ -convertase which then activates  $C_3$ - $C_9$  (ref. 1). To identify further the components upon which biological activity might depend, CVF was injected into  $C_6$ -deficient rabbits. Acute neutropenia followed by neutrophilia occurred, suggesting that the biological activity was dependent on either  $C_3$  and/or  $C_5$  (data not shown).

Further studies were then designed to further characterise the active factor. Fresh plasma (15 ml) was treated with either CVF (1:10 v/v) or 6 mg ml<sup>-1</sup> of inulin suspension. The mixtures were reacted for 15 min at 37° C and immediately filtered at 4° C through a UME 20,000 Amicon Diaflo filter. To enhance the filtration the plasma was first diluted 1:1 with sterile, isotonic saline. The filtrate was collected and then filtered through a 0.4  $\mu$ m Millipore filter before infusing it into the rabbit ear vein. Appropriate saline controls were performed.

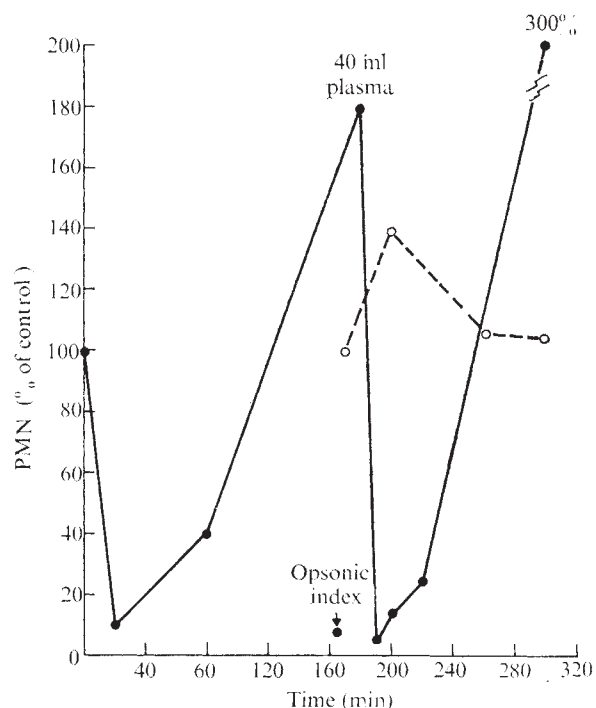
Figure 4 shows that the biological activity could be recovered in the filtrate. The small pore size would exclude CVF (molecular weight >180,000) and the  $C_3b$  immune adherence molecule (molecular weight ~150,000). The low molecular weight fraction recovered after treatment of plasma with CVF or inulin caused a biphasic curve following infusion. There is no explanation for this difference from *in vivo* administration of CVF or inulin. Low molecular weight filtrate produced very little neutropenia when the plasma treated *in vitro* with CVF was obtained from an animal first depleted of  $C_3$  by sequential doses of CVF. There was, however, pronounced neutrophilia, suggesting that neutrophilia follows activation of less substrate or that it is determined by a different mechanism.

Preliminary studies suggest that the low molecular weight factor acts directly on PMN and that the cells are removed by sequestration rather than lysis (our unpublished observations).

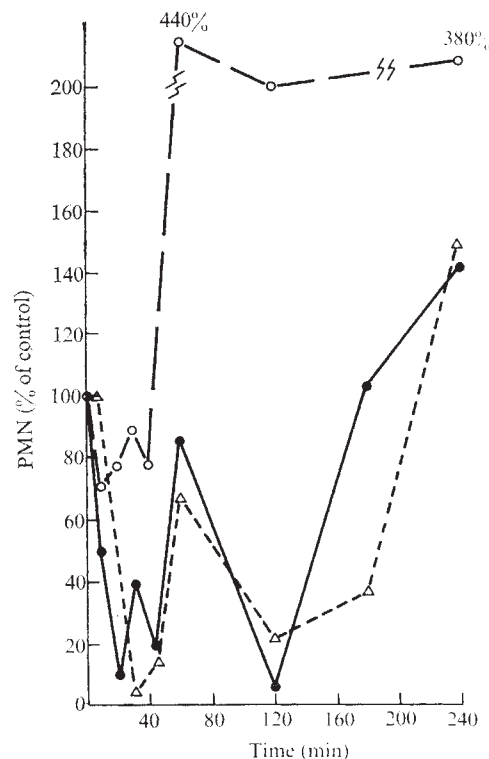
Alterations in neutrophil kinetics during acute inflammatory states have interested investigators for some time. Some of the most intriguing early observations were made by Andrewes<sup>6</sup>. This investigator demonstrated in rabbits that acute neutropenia followed intravenous administration of the live Gram-negative bacteria, and antigen-antibody complexes. Neutrophilia occurred later. Reactions to Gram-positive bacteria were mild unless animals were previously



**Fig. 2** Effect of two sequential doses of 1.0 ml CVF on antigenic  $C_3$  levels of serum (●) and zymosan opsonic index (○). The first dose of CVF was given at zero time and the second at 160 min. Antigenic  $C_3$  levels were measured by radial immunodiffusion using goat anti-rabbit  $C_3$  (ref. 3). The opsonic index for zymosan was obtained by counting the number of intracellular zymosan particles in 100 PMN (100 particles: 1 PMN) after incubating in a shaker bath at 37° C for 15 min.



**Fig. 3** Effect of infusion of 40 ml of fresh plasma in a rabbit which received 1.0 ml of CVF at zero time (●). The opsonic index for zymosan at the time of plasma infusion was 8% of control. A saline control in a normal rabbit is included (○).



**Fig. 4** Effects of infusion of 15 ml of low molecular weight filtrate (< 20,000) on rabbit PMN counts. Before filtration for approximately 2 h through an Amicon Diaflo UME 20,000 filter, 15 ml of fresh plasma was incubated with either CVF (1:10 v/v) or inulin 6 mg ml<sup>-1</sup> plasma. The plasma was diluted 1:1 with saline before filtration in order to increase the rate of filtration. ●, Filtrate from CVF treated plasma; △, filtrate from inulin-treated plasma; ○, filtrate from CVF treated plasma obtained from a rabbit that had received for sequential doses of CVF (0.7 ml) which reduced the antigenic  $C_3$  level to 20% of control before the time plasma was collected.

immunised. Most PMN were sequestered in the lung and there was no evidence that their destruction was the cause of the profound neutropenia. Interpreted in the light of contemporary knowledge, these early studies suggest that the acute neutropenia may have been complement dependent. With Gram-negative bacteria, the complement cascade would be activated by endotoxin<sup>1</sup>. The reaction to Gram-positive bacteria would be mild unless it was enhanced by the presence of specific antibody, after which the complement cascade would be activated by the antigen-antibody complex<sup>1</sup>. Moreover, both neutropenia and neutrophilia may occur in humans during acute bacterial infection, and the shock syndrome of infection is associated with activation of the complement cascade<sup>7</sup>. Neutropenia follows the injection of endotoxin into both humans and experimental animals and during this time the cells are primarily sequestered in the pulmonary capillaries<sup>8</sup>. Recent studies have indicated that the neutropenia induced after infusion of viable Gram-negative bacteria into the blood of subhuman primates is diminished if the animals are first depleted of the complement by CVF (ref. 9). In our investigation the possibility of contamination of the CVF, inulin or low molecular weight fraction by endotoxin was considered. This was excluded by constant temperature measurements for up to 3 h in rabbits receiving each of these. No significant alteration in rectal temperature was observed in any animal, thus making contamination with endotoxin highly unlikely.

A neutrophilia has been observed several hours after the infusion of CVF, but careful studies of time points earlier than 1 h were not done<sup>5,10</sup>. The magnitude of the neutrophilia was somewhat less than we observed and the reasons for this are not evident at present. A possible association of leukocytosis with complement activation has been recorded by Rother who observed that factors released during complement activation induce release of leukocytes from bone marrow<sup>11</sup>.

This investigation indicates that the alternate pathway activation of the fluid phase by CVF or by inulin is associated with alterations in PMN kinetics. The changes depend on elaboration of a factor of low molecular weight (< 20,000) which may be derived from either the C<sub>3</sub> or C<sub>5</sub> molecule. This factor may be responsible for alterations in PMN during acute inflammation.

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Received February 8; revised April 4, 1974.

## Evidence that most noradrenaline is stored without ATP in sympathetic large dense core nerve vesicles

THE concept that catecholamine (CA) in sympathetic nerve vesicles is stored together with ATP, in a fixed molar ratio of 4/1, is so widely accepted that it is even quoted in general textbooks on physiology and pharmacology for medical students<sup>1-3</sup>. This fixed ratio of CA to ATP has been convincingly demonstrated in purified preparations of adrenomedullary vesicles<sup>4,5</sup>, whereas the evidence concerning the ratio of noradrenaline (NA) to ATP in sympathetic nerve vesicles has been based on preparations from bovine splenic nerves<sup>6-9</sup>, rat heart<sup>10</sup> and rat vas deferens<sup>11</sup> contaminated to an unspecified extent with other organelles such as mitochondria. The aim of the present study was to determine the molar NA/ATP ratio in a highly purified preparation of bovine splenic nerve vesicles.

Large dense core vesicles were isolated from bovine splenic nerves using differential and density gradient centrifugation<sup>12,13</sup>. The supernatant obtained after centrifugation of the nerve homogenate at 10,500g<sub>max</sub> for 10 min was layered on sucrose-D<sub>2</sub>O gradients (linear from 0.25 M to 0.6 M with a step of 1.2 M in the bottom). The gradients were spun at 280,000g<sub>max</sub> for 90 min in a SW 40 rotor (Beckman). The band just above the bottom step (fraction III) in the gradients was taken out, diluted three-fold with isotonic sucrose and centrifuged at 10,500g<sub>max</sub> for 10 min. The supernatant obtained was centrifuged at 20,000g<sub>max</sub> for 10 min and the supernatant from this centrifugation was spun at 230,000g<sub>max</sub> for 20 min.

Each pellet was resuspended in 1.5 ml 20 mM K-phosphate buffer (pH 7.3); 0.5 ml was used for determination of cytochrome oxidase as a mitochondrial marker enzyme<sup>14</sup>. The rest (1 ml) was extracted with 0.4 M perchloric acid and then diluted with distilled water to 2 ml. After removal of the protein precipitate, 1 ml was used for fluorimetric determination of NA (ref. 12) and the remaining 1 ml was titrated with Tris-buffer and KOH to pH 7.0-7.4. The perchlorate precipitate was carefully removed by centrifugation. The supernatant was used for ATP assay by the firefly method<sup>15</sup>. The recovery of ATP added as internal standard ranged from 59-73%.

The ATP values were corrected for the loss actually measured in each individual experiment. The protein precipitate was solubilised in NaOH and protein was determined by the Folin method.

NA storage particles and mitochondria from splenic nerve sedimented to about the same level in the density gradients<sup>12</sup>. But a separation of NA storage particles and mitochondria could be achieved by differential centrifugation of the diluted gradient fraction as shown in Fig. 1. Cytochrome oxidase activity was highest in the first two pellets obtained after low speed centrifugations, while most of the NA was found in the last pellet which was obtained after high speed centrifugation. In this way about 80% of the cytochrome oxidase activity could be removed while still more than 60% of the NA was retained in the particles.

The high speed sediment with the lowest cytochrome oxidase activity was used for determination of the NA/ATP ratio. In the first few experiments there was a great variability in NA/protein ratio due to variable *post mortem* delay at the slaughter house before the nerves could be chilled with ice. When special precautions were taken to shorten the delay to 15-20 min, the average NA/protein ratio was  $5.30 \pm 0.74 \mu\text{g mg}^{-1}$  (s.e.m.,  $n = 11$ ) and the molar NA/ATP ratio  $7.32 \pm 0.30$ .

The original density gradient fraction (fraction III) consisted mainly of large dense core vesicles (750 Å), contaminated almost exclusively with mitochondria according to biochemical<sup>12</sup> and morphological analyses<sup>16</sup>. As seen in Fig. 1 most of the mitochondria likely to contain appreciable amounts of ATP, could be effectively separated from the NA storage particles, as has also been confirmed by morphological studies<sup>16</sup>.

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<sup>3</sup> Lachmann, P. J., Hobart, N. J., and Aston, W. P., in *Experimental immunology*, second ed. (edit. by Weir, P. M.), (Blackwell, Oxford, in the press).

<sup>4</sup> Brown, D. L., dissertation, Univ. Cambridge (1972).

<sup>5</sup> Cochrane, C. G., Müller-Eberhard, H. J., and Akin, B. S., *J. Immun.*, **105**, 55 (1970).

<sup>6</sup> Andrewes, F. W., *Lancet*, **i**, 8 (1910).

<sup>7</sup> McCabe, W. R., *New Engl. J. Med.*, **288**, 21 (1973).

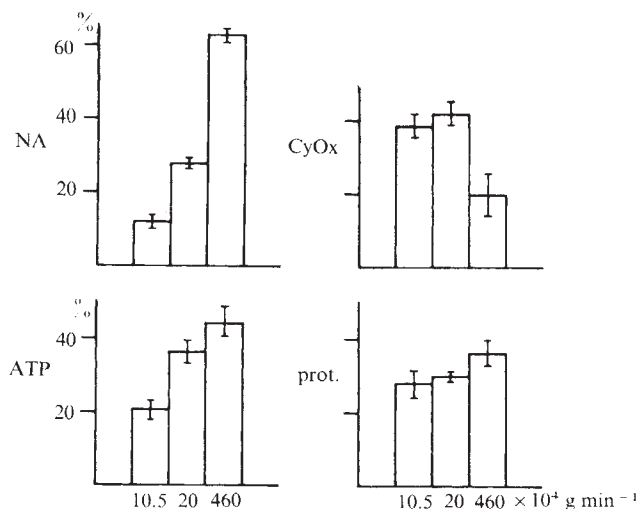
<sup>8</sup> McKay, D. G., *Thrombos. Diathes. Haemorrh.*, **22**, 11 (1973).

<sup>9</sup> Gilbert, D. N., Barnett, J. A., and Sanford, J. P., *J. clin. Invest.*, **52**, 406 (1973).

<sup>10</sup> Dodds, W. J., and Pickering, R. J., *Blood*, **40**, 400 (1972).

<sup>11</sup> Rother, K., *Eur. J. Immun.*, **2**, 550 (1972).





**Fig. 1** Distributions of NA, ATP, cytochrome oxidase (CyOx) and protein (prot.) in the three sediments. These were prepared from a 'nerve vesicle fraction' (obtained from a density gradient after ultracentrifugation) by fractional centrifugations at 10,500  $g_{max}$  for 10 min; 20,000  $g_{max}$  for 10 min and 230,000  $g_{max}$  for 20 min. The values are expressed in percentages  $\pm$  s.e.m. Each bar represents the mean of at least four experiments.

The much more complete removal of cytochrome oxidase than of ATP indicates that the ATP sedimenting together with NA truly belongs to the NA storage particles. The possibility of contamination with mitochondrial fragments containing ATP but not cytochrome oxidase (outer membrane fractions) cannot be ruled out. But these particles would probably not sediment to the same level in the density gradients<sup>14</sup>.

It was not possible to get rid of all the cytochrome oxidase in the final sediment. According to the morphological studies<sup>16</sup> the mitochondria constituted a few per cent of all the particles. Therefore the NA/ATP ratio was certainly slightly higher than 7.3 in the NA storage particles in the final sediment.

We have not excluded the possibility that there were nerve vesicles with relatively higher ATP content which were lost during the first centrifugations. The overall NA/ATP ratio was however found to be 5.1/1 in the original preparation containing at least 10% mitochondria according to morphological analysis<sup>16</sup>, so it is quite possible that the NA/ATP ratio also was higher than 4/1 in the lost vesicles.

The 'true' *in vivo* ratio of NA to ATP may be even higher than the value 7.3 obtained in the present study. Since there is evidence that thermal activation leads to a selective loss of NA without concomitant loss of ATP<sup>8</sup>, it seems probable that *post mortem* delay at body temperature reduces the NA/ATP ratio of the vesicles. Thus, when the delay was 30–40 min the NA/protein ratio was about 2  $\mu$ g mg<sup>-1</sup> and the molar NA/ATP ratio was about 3.5. When the delay was reduced to about 10 min, vesicle preparations with a NA/protein ratio of 10  $\mu$ g mg<sup>-1</sup> could be obtained. The NA/ATP ratio in these preparations was 7–12.

Extrapolation to zero time<sup>13</sup> suggests that the NA/ATP ratio *in vivo* may be as high as 20/1 which would indicate that only 20% of the NA in these sympathetic nerve vesicles could be stored in the postulated NA/ATP complex. High values for the NA/ATP ratio have been observed previously; a level of 7.5–12 has been calculated to occur in sympathetic large dense core vesicles<sup>17</sup>. Moreover, in a series of studies on pheochromocytoma tumours a wide range of CA/ATP ratios were found, from 8 to as much as 35 (refs 18, 19). It may be of further interest that cholinergic nerve vesicles seem to store acetylcholine in a molar ratio to ATP of 10/1; and 5/1 in the two discernable pools<sup>20</sup>.

The occurrence of other nucleotides in sympathetic nerve vesicles cannot be ruled out, but only trace amounts of such material, including ADP, could be detected in partially purified splenic nerve vesicles (DaPrada, personal communication).

This study concerns the NA storage particles in nerve trunk. It is possible that the vesicles in the nerve terminals have a relatively higher content of ATP, corresponding to the proximo-distal increase in NA/protein ratio<sup>21</sup>. But preliminary studies do not support this possibility, since no significant rise was found in the ATP/protein ratio in the vesicles isolated from the intrasplenic distal part of the nerve trunk, compared with vesicles from the proximal part, while the NA/protein ratio increased markedly.

The present results provide very strong evidence that a major proportion of the NA in the sympathetic nerve vesicles is stored differently from the bulk of the CA in the adrenomedullary vesicles. This is further supported by the findings of striking differences in kinetic properties<sup>8,12</sup>; between nerve and adrenomedullary vesicles.

The nerve vesicles are, for example, very resistant to osmotic shock, but they lose NA rapidly during thermal activation, while the adrenomedullary vesicles behave in the opposite way<sup>8</sup>. Furthermore, the nerve vesicles have a very low content of soluble proteins such as chromogranin A compared with the chromaffin vesicles<sup>9</sup>. The nerve vesicles may contain a small NA/ATP/soluble protein complex of the same kind as that which predominates in adrenomedullary vesicles; it seems possible that this complex in nerve vesicles is functionally insignificant and may represent an evolutionary dead end. If ionically bound, most of the NA in nerve vesicles has to be bound by negatively charged constituents other than ATP such as phospholipids.

We thank I. Dahlin and I. M. Eriksson for technical assistance; Farmek, Uppsala for supplying the nerves and the Swedish Medical Research Council and Magnus Bergwalls Stiftelse for financial support.

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Received January 22; revised March 8, 1974.

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## (+) and (-)-bicuculline methochloride as optical isomers of a GABA antagonist

THE discovery by Curtis *et al.*<sup>1</sup>, that bicuculline would antagonise both the depressant effects of microiontophoretically applied  $\gamma$ -aminobutyric acid (GABA) and certain strychnine resistant central inhibitions has provided further support for the view that GABA might be a central transmitter. Their studies have also stimulated much thinking and research into the structural relationship of GABA and its receptor. On the basis of examination of Dreiding stereomodels of GABA and bicuculline, the Canberra group suggested that the nitrogen atoms and the three atoms associated with the carboxylate in GABA ( $O = C - O$ ) could be isosteric with the nitrogen atom and the  $O = C - C$  grouping in bicuculline<sup>1</sup>. Indeed, recent physicochemical work<sup>2</sup> on the two substances indicates that the distance from the onium group to a carboxylate oxygen in GABA is fixed at between 5.2 and 5.8 Å and this agrees well with a distance of 5.6 Å between the equivalent groups in bicuculline. It would therefore seem reasonable to suppose that the GABA antagonist properties of bicuculline are due to the occupation of the GABA receptor by a part of the bicuculline molecule that is isosteric with the biologically active conformation of GABA<sup>1</sup>.

Closer inspection of the portion of the bicuculline molecule that is isosteric with GABA reveals that it contains two asymmetric centres and thus four optical isomers are possible. Such variation in the optical properties would be unlikely to change interatomic distances along the skeleton of the molecule and so may not affect the potency as a GABA antagonist. If, on the other hand, the stereochemical requirements for combination with the GABA receptor are more subtle, then different optical isomers would be expected to have widely differing potencies. All pharmacological experiments so far performed with bicuculline<sup>3</sup> have used the commercially available (+) form (K & K Chemical, Pierce Chemical) and, so we thought it important to examine the properties of the (-) isomer. Although the (-) form occurs in nature<sup>4</sup> it is rare and we have therefore prepared it by synthesis from (-)- $\beta$ -hydrastine<sup>5</sup>.

In comparing the biological activities of the two stereoisomers of bicuculline certain problems arise, chiefly associated with their very low solubility at biological pH, and this has already led to dispute over the efficacy of the (+) isomer<sup>6,7</sup>. This would obviously lead to uncertainty over the correct interpretation of a negative effect with the (-) form. More recently (+) bicuculline methochloride has been shown to be an effective GABA antagonist and as this substance is about 100 times more soluble in water than bicuculline base, its use, particularly in microiontophoresis experiments, is easier and gives more reproducible results<sup>8</sup>. As the asymmetric centres are not affected by methylation we have chosen to prepare the methochlorides from our samples of (+) and (-)-bicuculline and to compare the relative biological activities of these derivatives.

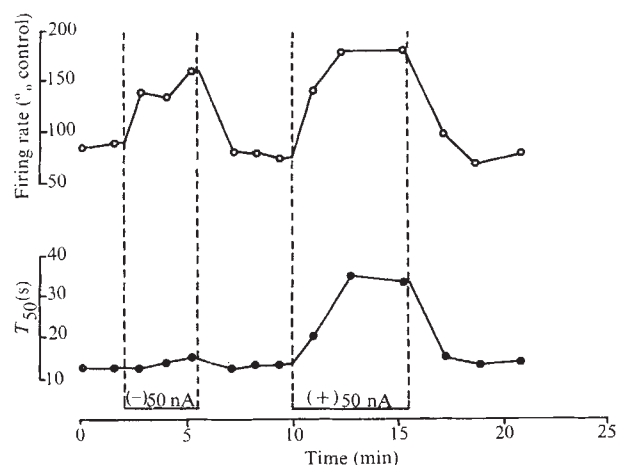
All experiments have been performed in piebald Lister rats anaesthetised with urethane, and respiring spontaneously. Microiontophoretic techniques were as previously described<sup>9</sup> and all recordings of unit activity were extracellular. Neurones were located in the cerebral cortex and cuneate nucleus, and (+)-bicuculline methochloride (10–50 nA from 5 mM solution in 150 mM saline) consistently

antagonised the depressant action of GABA on these neurones and produced concurrent excitation. (-)-Bicuculline methochloride (prepared in a similar solution, 10–100 nA) applied to the same neurones, from the same pipette, was ineffective as a GABA antagonist but produced neuronal excitation in a similar manner to the (+) isomer (Fig. 1).

Presynaptic inhibition was measured in the cuneate nucleus by analysis of the evoked potential recorded there in response to stimulation of a forepaw ipsilateral to the recording electrode. The amplitude of the positive-going potential (P wave) was taken as an index of presynaptic terminal depolarisation<sup>10</sup>. (+)-Bicuculline methochloride (0.5 mM solution in normal saline), applied topically to the medulla, consistently and reversibly antagonised presynaptic inhibition in the nucleus, whereas (-)-bicuculline methochloride (4 mM solution in normal saline) was without effect on the P wave (Fig. 2).

The relative convulsant potencies of the two isomers were tested by making microinjections into cerebral cortex and recording the resultant convulsive discharges in the EEG, the injection cannula serving as recording electrode. Very small quantities of the (+)-bicuculline methochloride produced sustained convulsive activity ('spiking') (2  $\mu$ l of 0.5 mM solution in normal saline) whereas injection of 400 times this amount of the (-) form to the same animal (10  $\mu$ l of 4 mM solution in normal saline) produced only transient convulsive activity.

More recently, experiments in the cat have shown that (+)-bicuculline methochloride will readily antagonise both GABA and synaptic inhibition in the cuneate nucleus, but that (-)-bicuculline methochloride does neither. Both isomers seemed to have little effect on depression of these neurones by glycine<sup>11</sup>. In comparison with other GABA antagonists that we have studied, (+)-bicuculline methochloride stands out for both its potency and its lack of effect on glycine (R. G. H. and M. A. Simmonds, in preparation).



**Fig. 1** Production of an increase in firing rate of a single cortical neurone by both (+) and (-)-bicuculline methochloride and GABA antagonism by the (+) isomer. The firing of this neurone was assisted with a continuous application of *D,L*-homocysteate 5 nA. Firing rate (O) immediately preceding each GABA application is plotted against elapsed time. The time taken to achieve 50% depression of firing ( $T_{50}$ ) (●), by successive application of GABA 40 nA, measures the effectiveness of the GABA<sup>9</sup>. Each point represents one GABA application. Where shown by the first horizontal bar (-)-bicuculline methochloride 50 nA was applied and produced an increase in the firing rate, although it did not affect the response to GABA. The second horizontal bar represents an application of (+)-bicuculline methochloride 50 nA and this produced a similar increase in firing rate. Additionally the GABA  $T_{50}$  was much prolonged, indicating GABA antagonism had occurred. Both effects were readily reversible.

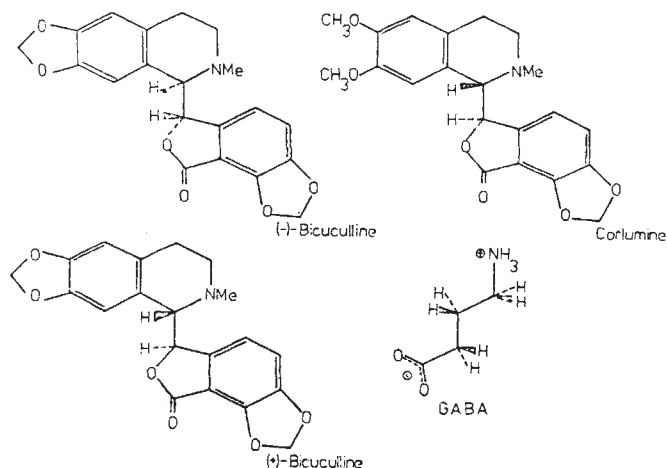


Thus, from these experiments, it would appear that only the (+) form of bicuculline methochloride is capable of antagonising both the depressant effects of GABA and presynaptic inhibition. Additionally, (+)-bicuculline methochloride is at least 400 times as potent a convulsant as (-)-bicuculline methochloride. It would, therefore, seem that the biological activity of these compounds is dependent on the absolute configuration around the asymmetric centres.

The GABA molecule is flexible and can exist in many different conformations. Attempts have been made<sup>1,12,13</sup>, using molecular models, to explain the GABA antagonist action of bicuculline in terms of a stereochemical match with GABA. Blount and Gilardi have recently, independently determined the absolute configuration of bicuculline using X-ray analysis and Gilardi has pointed out that the previous model studies<sup>1,12,13</sup> on the preferred conformations for GABA and bicuculline were probably based on an incorrect configuration for bicuculline. Some workers<sup>2,14-16</sup> have suggested using molecular orbital and NMR calculations for protonated bicuculline and bicuculline methochloride in solution, as a way of determining the correct preferred conformations. Our results suggest that whatever the preferred conformations of bicuculline and bicuculline methochloride are found to be, the conformation of the (+) isomer will be more correctly isosteric with the preferred biologically active conformation of GABA than will the (-) enantiomer. This stereospecificity parallels that seen elsewhere in the nervous system, for example with muscarinic agonists of acetylcholine, narcotic analgesics and their antagonists and  $\beta$ -receptor blocking agents.

(+)-Bicuculline methochloride (Fig. 3) possesses the 1S, 9R configuration<sup>17</sup>, whereas (-)-bicuculline methochloride (Fig. 3) has the 1R, 9S configuration<sup>17</sup>, these configurations of course, being shared by the parent compounds (+) and (-)-bicuculline. These observations are compatible with the work of Johnston and his co-workers who revealed that corlumine (Fig. 3) is an effective GABA antagonist<sup>8</sup> for on examination of the stereochemistry we found that this substance also had the 1S, 9R configuration.

We are now examining the biological activity of bicuculline epimers and related compounds to determine whether the particular stereochemical arrangement involv-



**Fig. 3** Structural formulae showing the configurations of (+) and (-)-bicuculline and corlumine about their asymmetric centres. GABA is shown for comparison in one of its possible conformations. Although precise conformational information is not yet available, the preferred biologically active conformation of GABA is more likely to resemble the configurations of the isosteric groupings in (+)-bicuculline. Although the conformation of (+) and (-)-bicuculline methochloride will not be precisely that of (+) and (-) bicuculline due to steric effects of the extra methyl group<sup>14,15</sup>, the configuration about the asymmetric centres will be identical.

ing a 1S, 9R configuration is the unique criterion for GABA antagonism in this series of alkaloids.

During this work J.F.C. was the Sir Harry Jephcott Research Fellow and R.G.H. was supported by the bequest of W. L. Custance. We thank Celia Button for technical assistance and Professors D. W. Straughan and W. B. Whalley for laboratory facilities. This work was supported in part by a Medical Research Council programme grant to D.W.S.

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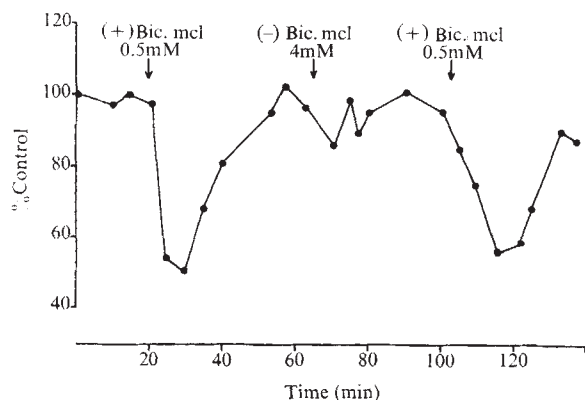
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**Fig. 2** Reduction of presynaptic inhibition in the rat cuneate nucleus by (+)-bicuculline methochloride and the lack of effect of the (-) isomer. The inhibition was measured as the amplitude of the P wave (expressed as percentage of control) recorded in the cuneate in response to peripheral stimulation. At the first arrow (+)-bicuculline methochloride 0.5 mM solution in saline was applied to the surface of the medulla and rapidly reduced the P wave amplitude. After 40 min the P wave amplitude had returned to control values and application of 4 mM (-)-bicuculline methochloride solution at the second arrow had a negligible effect. A subsequent application of the (+) isomer (third arrow) again readily and reversibly depressed the P wave.

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## Spin-lattice relaxation times of imidazole protons and their relevance to NMR studies of proteins

THE spin lattice relaxation time ( $T_1$ ) is the exponential time constant for re-equilibration of nuclear spins with their surroundings (the lattice) following resonance excitation, and is consequently related to the correlation time of the nuclei in solution. Thus, the determination of  $T_1$  values would be of interest in many biological systems. Measurement of the relative motility of different portions of a protein molecule, for example, could shed light on conformational and functional properties.

Recent technical innovations, notably the introduction of the Fourier transform (FT) technique in nuclear magnetic resonance (NMR) (ref. 1) have made determinations of  $T_1$  almost routine. There are several reports of the measurement of  $T_1$  values of imidazole  $C_2$  protons of histidine residues in peptides and proteins<sup>2-5</sup>, as well as applications of  $^{13}\text{C}$  NMR (refs 6-11). Previous work has indicated the value of measuring the chemical shift of the imidazole proton resonances as a function of pH (ref. 12 and ref. 13 and refs therein). We now report the  $T_1$  values of imidazole protons (Figs 1 and 2) as a function of pH.

The  $T_1$  values of both the  $C_2$  and  $C_{4,5}$  protons of fresh solutions of imidazole (0.073 M) in 0.1 M sodium chloride in deuterium oxide, showed a minimum at about neutral pH (Fig. 3). Measurements made in the absence of (paramagnetic) oxygen by freeze-thawing five times under high vacuum then sealing the tubes, showed an even more pronounced minimum in the data (Fig. 3). Similar results were also observed for histidine, including degassed samples (Table 1). Increasing the salt concentration by a factor of ten in non-degassed samples of imidazole resulted in no significant change. The imidazole concentration was also decreased by a factor of ten and the absence of any change eliminated the possibility of intermolecular association as the origin of the pH dependence (Fig. 3).

Values measured in the presence of  $10^{-4}$  M ethylenediamine tetra-acetate (EDTA) increased at neutral pH (Table 1 and Fig. 4). By contrast, the addition of  $10^{-5}$ – $10^{-6}$  M  $\text{Cu}^{2+}$  resulted in an expected lowering of the  $T_1$  values of the ring protons at neutral pH, but had less effect in acidic or basic solution (Table 1 and Fig. 4). These results indicate that the phenomenon of the minimum in the pH dependence of the  $T_1$  values of the imidazole protons arises from the binding of paramagnetic metal ion impurities in the samples at concentrations of  $10^{-7}$  M or below. At low pH these ions would be displaced by deuterons and at high pH would bind more strongly to deuteride ions. The  $T_1$  value of  $\text{H}_2\text{O}$  is reported to show no pH dependency<sup>14,15</sup>. (No significant pH dependence was observed for the  $T_1$  value of HDO in the present work, even in the presence of  $7 \times 10^{-5}$  M  $\text{Cu}^{2+}$  ion, as expected<sup>16</sup>.)

If the pH dependence of  $T_1$  values were to be explained by a change in the correlation time ( $\tau_c$ ) of the imidazole ring at different pH values, this would require, for example, a fifteen-fold change in  $\tau_c$  of the imidazole  $C_2$  proton between pH 1.4

**Table 1** Spin-lattice relaxation times ( $T_1$ ) of imidazole protons

| Sample | pH   | Conditions*             | $T_1$ s           |              |
|--------|------|-------------------------|-------------------|--------------|
|        |      |                         | C2H               | C4H          |
| His    | 0.0  | Degassed                | 18 ± 1.0          | 9.9 ± 0.5    |
|        | 4.6  | Degassed                | 8.1 ± 0.5         | 7.0 ± 0.5    |
|        | 11.5 | Degassed                | 24 ± 2.0          | 15.6 ± 2.0   |
| Im A   | 6.77 | —                       | 5.04 ± 0.03       | 6.57 ± 0.03  |
|        | 6.77 | EDTA ( $10^{-4}$ M)     | 12.4 ± 0.1        | 13.2 ± 0.1   |
| Im B   | 7.1  | —                       | 6.01 ± 0.14       | 8.19 ± 0.03  |
|        | 7.1  | EDTA ( $10^{-4}$ M)     | 12.6 ± 0.2        | 15.5 ± 0.2   |
| Im C   | 7.1  | EDTA                    | 14.4 ± 0.1        | 18.8 ± 0.1   |
|        | 9.46 | Degassed                | 2.21 ± 0.07       | 3.14 ± 0.05  |
| Im D   | 9.46 | EDTA ( $10^{-4}$ M)     | 15.79 ± 0.21      | 14.62 ± 0.16 |
|        | 0.7† | $\text{Cu}^{2+}$        | —                 | —            |
| Im E   | 3.43 | ( $7 \times 10^{-5}$ M) | 21.9 ± 1.8        | 16.2 ± 0.2   |
|        | 3.43 | $\text{Cu}^{2+}$        | 21.3 ± 0.3        | 18.1 ± 0.2   |
| Im F   | 7.1  | ( $7 \times 10^{-6}$ M) | 10.6 ± 0.2        | 11.6 ± 0.2   |
|        | 7.1  | $\text{Cu}^{2+}$        | 1.5 ± $10^{-5}$ M | 0.35 ± 0.02  |
| Im G   | 7.1  | ( $3 \times 10^{-5}$ M) | 0.20 ± 0.08       | 0.37 ± 0.05  |
|        | 0.7  | Im Dil × 10             | 33.1 ± 0.7        | 31.1 ± 0.8   |
| Im I   | 3.3  | EDTA ( $10^{-3}$ M)     | —                 | —            |
|        | 6.9  | Degassed                | 31.8 ± 1.2        | 26.6 ± 0.6   |
| Im J   | 6.9  | Degassed                | 0.83 ± 0.04       | 1.51 ± 0.02  |
|        | 7.7  | Degassed                | 1.96 ± 1.1        | 3.14 ± 0.18  |

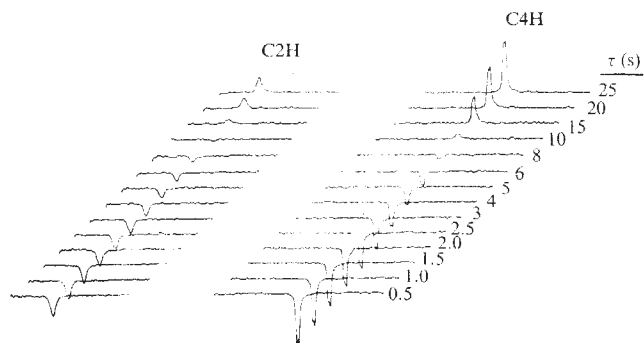
\* Unless otherwise stated imidazole is 0.073 M in 0.1MNaCl– $\text{D}_2\text{O}$ . Most values for imidazole shown in this table are not given in Figs 3 and 4.

† At pH 7.1 the C2 and C4 proton lines are extremely broad in the presence of  $7 \times 10^{-5}$  M  $\text{Cu}^{2+}$ .

and 7.1 in the degassed samples. The viscosity of water is relatively insensitive to pH, and such a large change in  $\tau_c$  is considered unlikely.

Estimates of the  $T_1$  values for a nucleus can be made on the assumptions that only intramolecular dipole-dipole interactions are effective, and that rapid isotropic motion is occurring<sup>1,2,5</sup>. Since the  $C_4$  and  $C_5$  protons effectively relax each other, the  $T_1$  values for these protons in acid pH are about half those of the  $C_2$  proton. At neutrality, however, the ratio of the  $T_1$  values is reversed (Table 1). This implies that the relaxation times are dominated by the paramagnetic ions present, and that these spend more time on average closer to the  $C_2$  proton than the  $C_{4,5}$  protons.

Calculations were made of the effect of protonation of the imidazole ring on the  $T_1$  values<sup>17</sup> using the geometry determined by X-ray crystallography<sup>18</sup>. Results indicated only a small



**Fig. 1** Spectra of imidazole (0.073 M in 1.0 M NaCl, pH 2.0) at 220 MHz recorded on a Varian 220 FT spectrometer at  $20 \pm 1^\circ\text{C}$ . A single ( $d$ ,  $180^\circ$ ,  $t$ ,  $90^\circ$ ) pulse sequence was used, where  $d$  is a delay time ( $d > 5T_1$ ) and  $t$  is a variable time. At short values of  $t$  the spins are recorded as essentially inverted by the  $180^\circ$  pulse, while at long values of  $t$  they approach their maximum intensity value ( $100\%$ ). The  $T_1$  values determined in this case were  $C_2\text{H}$ ,  $20.6 \pm 0.30$  s;  $C_{4,5}\text{H}$ ,  $17.6 \pm 0.30$  s. Values of pH are direct meter readings in  $\text{D}_2\text{O}$  (ref. 12).



change in the  $T_1$  value of both the  $C_2$  and  $C_{4,5}$  protons would be expected on protonation of the ring assuming  $\tau_c$  the same in both cases. This effect would probably be comparable with errors in the  $T_1$  measurements. The increase observed in the  $T_1$  values at low pH for the imidazole protons in histidine seems to have been mistakenly interpreted to indicate the titration of the ring<sup>3</sup>, rather than the pH dependence of the binding of paramagnetic impurities.

Sodium chloride was used in these experiments to act as a reservoir of ionic strength to avoid change in this parameter when pH changes. Similar experiments with proteins would require a salt or buffer for reasons of solubility. Thus, the levels of impurity observed in these experiments are not above a reasonable value for practical applications of this technique. Analyses of highly purified imidazole grade III (Sigma) by atomic absorption gave  $0.17 \times 10^{-7}$  mg  $\text{Cu}^{2+}$   $\text{mg}^{-1}$  imidazole or  $0.12 \times 10^{-8}$  M  $\text{Cu}^{2+}$ . The sodium chloride used in these experiments was Baker Analyzed reagent, with a specified heavy metal analysis including iron of 0.0002%. Analysis of the copper content in the standard 0.1 M NaCl- $\text{D}_2\text{O}$  solution used gave a value of  $2 \times 10^{-5}$  mg  $\text{Cu}^{2+}$   $\text{ml}^{-1}$ , corresponding to  $8 \times 10^{-8}$  M. Low temperature electron spin resonance (ESR) spectra of the solid NaCl and imidazole showed negligible amounts of paramagnetic material present. Further, the use of 1.0 M NaCl gave only a slight decrease in the  $T_1$  values at neutral pH (Fig. 3) indicating that the extra NaCl did not provide a substantial increase in the level of paramagnetic impurity above the background range of approximately  $10^{-7}$  M.

In view of the effects of degassing (Fig. 3) and EDTA (Fig. 4) separately on the  $T_1$  values of the imidazole protons, experiments were performed by degassing in the presence of  $10^{-4}$  M EDTA. As expected,  $T_1$  values of the imidazole protons were increased, but there was still a minimum in the pH dependence (Fig. 4). This implies that the EDTA does not completely scavenge metal ions, allowing partial interaction with imidazole at neutral pH. Indeed there is evidence for the formation of a ternary copper-EDTA-imidazole complex<sup>19</sup>, as for many other such combinations<sup>20,21</sup>. To test whether such a complex was being formed in our experiments, we reduced the ratio of concentrations of imidazole:EDTA by two orders of magnitude. If EDTA was

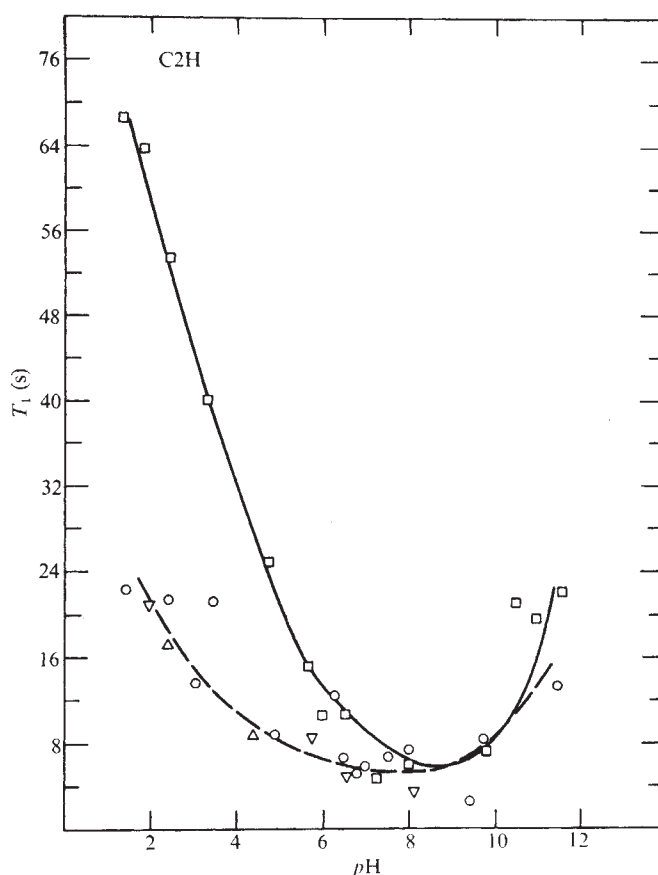


Fig. 3 Plot of the spin-lattice relaxation time ( $T_1$ ) against pH for the  $C_2$  proton of imidazole. Unless otherwise shown, all measurements were carried out in 0.1 M NaCl- $\text{D}_2\text{O}$  in undegassed samples. The curves are hand-drawn indications of the pH dependence. The standard error in the  $T_1$  determinations (Fig. 2) varied with the value obtained, for example, for the degassed samples for  $C_2\text{H}$ , pH 1.40,  $T_1$   $66.5 \pm 0.7$  s; 6.07,  $10.6 \pm 0.4$  s; 10.50,  $20.8 \pm 0.6$  s.  $\square$ , Degassed;  $\circ$ , Undegassed;  $\triangle$ , diluted ten times;  $\nabla$ , 1.0 M NaCl.

effectively scavenging metal ions the  $T_1$  values of the imidazole protons would be expected to increase. But if a ternary complex was being formed, the  $T_1$  values would be expected to decrease. In fact, a significant decrease in the  $T_1$  values was observed for  $7 \times 10^{-3}$  M imidazole with  $10^{-3}$  M EDTA (Table 1) in degassed samples, supporting the formation of a ternary complex.

The sensitivity of spin-spin relaxation times ( $T_2$ ) of purine proton resonances to impurities of copper ion at concentrations of  $10^{-6}$  to  $10^{-5}$  M has recently been emphasised<sup>22-24</sup>. In the light of such considerations,  $T_2$  evaluations from line width studies, particularly as a function of pH, must be treated with caution, except where the binding of paramagnetic ion well beyond the impurity level is being studied<sup>25</sup>. Calculations of  $T_2$  from the line widths of the imidazole proton resonances in the presence of  $\text{Cu}^{2+}$  in this work show that  $T_1 \approx 10 T_2$ . Thus, calculations based on the common assumption that  $T_1 \approx T_2$  for such systems are probably in error. An increase in the line widths of imidazole proton resonances at intermediate pH values commonly observed may result at least in part from the pH dependence of the ion binding reported here, rather than the pH dependence of the protonation rate<sup>26,27</sup>. Thus, the interpretation of line widths of resonance in proteins in terms of structural effects<sup>28</sup> may be difficult.

$T_1$  values reported for the  $C_2$  proton of imidazole residues in proteins have been interpreted to be due to the close approach of protons on other residues in the folded tertiary conformation of the native structure<sup>2,3</sup>. Due to the reciprocal relationship

$$(1/T_1)_{\text{obs}} = (1/T_1)_{\text{intrinsic}} + (1/T_1)_{\text{para}}$$

the effect of paramagnetic species would be expected to be greater on protons with intrinsically longer, rather than shorter,

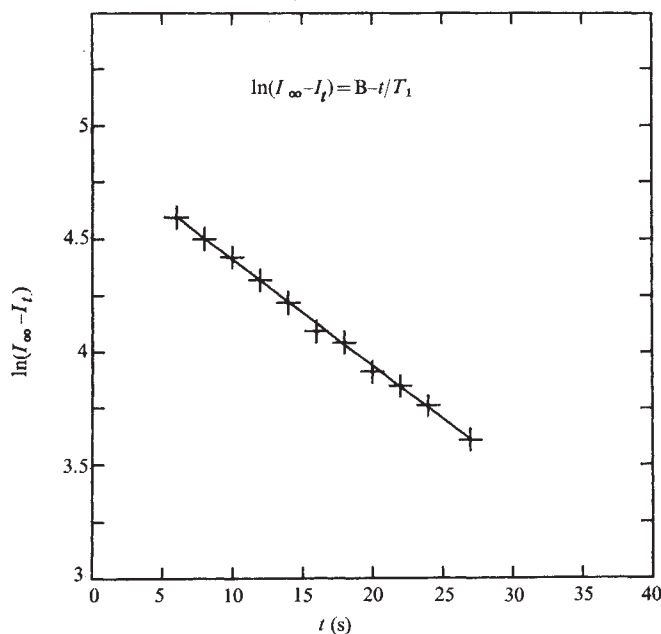


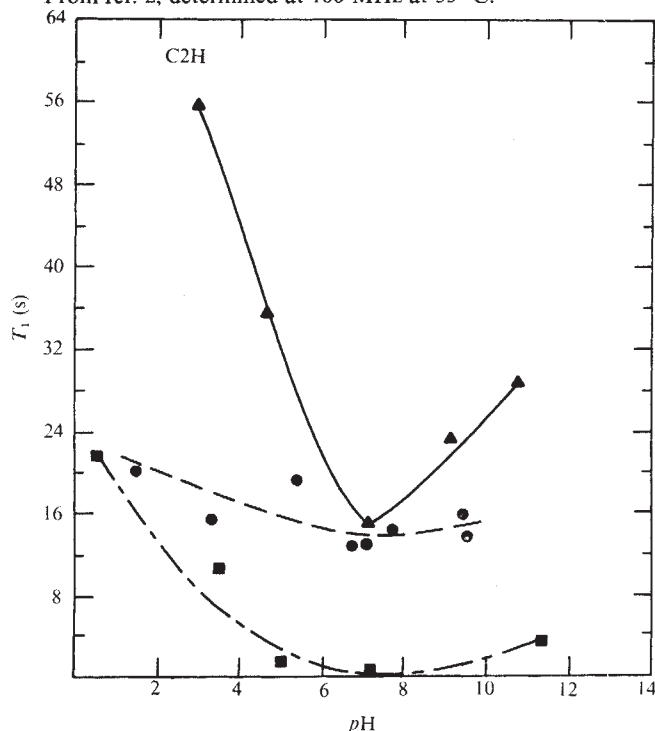
Fig. 2 Plot of the log of the intensity difference at time  $t$  ( $I_{\infty} - I_t$ ) against  $t$  derived as in Fig. 1. The equation provides the best straight-line fit to the data, the slope of the line then gives the value of  $T_1$ . The value of the intercept  $B$  theoretically equals  $\ln(2I_{\infty})$ . Imidazole pH = 3.43;  $T_1 = 21.29 \pm 0.32$ .

**Table 2** Spin-lattice relaxation times ( $T_1$ ) of imidazole protons of histidine residues in several proteins

| Protein                | Histidine residues | Salt                             | pH   | $T_1$ (s)       |
|------------------------|--------------------|----------------------------------|------|-----------------|
| Hen egg white lysozyme | 15                 | 0.1 M NaCl                       | 1.4  | $0.69 \pm 0.02$ |
|                        |                    | 0.1 M NaCl                       | 3.6  | $0.93 \pm 0.03$ |
|                        |                    | 0.1 M NaCl                       | 4.3  | $0.90 \pm 0.03$ |
|                        |                    | 0.1 M NaCl                       | 6.0  | $0.82 \pm 0.06$ |
| Human lysozyme         | 78                 | 0.1 M NaCl                       | 3.0  | $2.65 \pm 0.07$ |
|                        |                    | 0.1 M NaCl                       | 4.5  | $2.41 \pm 0.12$ |
|                        |                    | 0.1 M NaCl                       | 6.0  | $1.33 \pm 0.04$ |
|                        |                    | 0.1 M NaCl                       | 1.7  | $0.88 \pm 0.05$ |
| RNase A                | 105 (H-1)          | 0.1 M NaCl                       | 3.4  | $1.02 \pm 0.04$ |
|                        |                    | 0.1 M NaCl                       | 6.0  | $1.07 \pm 0.06$ |
|                        |                    | 0.2 M                            | 5.7  | $1.09 \pm 0.02$ |
|                        |                    | CD <sub>3</sub> COONa 0.2 M      | 5.5* | $0.77 \pm 0.03$ |
|                        | 12 (H-2)           | CD <sub>3</sub> COONa 0.1 M NaCl | 1.7  | $0.76 \pm 0.02$ |
|                        |                    | 0.1 M NaCl                       | 3.4  | $0.42 \pm 0.02$ |
|                        |                    | 0.1 M NaCl                       | 6.0  | $0.39 \pm 0.04$ |
|                        |                    | 0.2 M                            | 5.7  | $0.92 \pm 0.04$ |
|                        | 119 (H-3)          | CD <sub>3</sub> COONa 0.2 M      | 5.5* | $0.28 \pm 0.02$ |
|                        |                    | CD <sub>3</sub> COONa 0.1 M NaCl | 1.7  | $0.72 \pm 0.05$ |
|                        |                    | 0.1 M NaCl                       | 3.4  | $0.41 \pm 0.02$ |
|                        |                    | 0.1 M NaCl                       | 6.0  | $0.82 \pm 0.04$ |
|                        | 48 (H-4)           | 0.2 M                            | 5.7  | $1.07 \pm 0.03$ |
|                        |                    | CD <sub>3</sub> COONa 0.2 M      | 5.5* | $0.40 \pm 0.02$ |
|                        |                    | CD <sub>3</sub> COONa 0.1 M NaCl | 1.7  | $1.03 \pm 0.09$ |
|                        |                    | 0.1 M NaCl                       | 3.4  | $1.07 \pm 0.04$ |
|                        |                    | 0.1 M NaCl                       | 6.0  | Not observed    |
|                        |                    | 0.2 M                            | 5.7  | $1.14 \pm 0.07$ |
|                        |                    | CD <sub>3</sub> COONa 0.2 M      | 5.5* | $0.51 \pm 0.03$ |
|                        |                    | CD <sub>3</sub> COONa            |      |                 |

Values were calculated as shown in Fig 2. It is not easy to measure  $T_1$  values of C<sub>2</sub> protons in proteins at pH > 6 due to the poor baseline from adjacent resonances. The four C<sub>2</sub> proton resonances in RNase A are resolved at only a few pH values. Assignments of the resonances (numbered H-1 to H-4 at pH 5.5) are taken from ref. 34.

\* From ref. 2, determined at 100 MHz at 33° C.



**Fig. 4** Plot of the spin-lattice relaxation time ( $T_1$ ) against pH for the C<sub>2</sub> proton of imidazole in the presence of EDTA and Cu<sup>2+</sup> ion. ▲, EDTA 10<sup>-4</sup>M degassed; ●, EDTA 10<sup>-4</sup>M undegassed; ■, Cu<sup>2+</sup> 7 × 10<sup>-6</sup>M.

$T_1$  values. We have measured  $T_1$  values of imidazole resonances in several proteins (Table 2), and have observed values as long as two seconds for the single histidine residue present in human lysozyme. Indeed, the pH dependence in this case showed an increase in acid pH similar to the phenomenon seen in imidazole itself. The values obtained for bovine pancreatic RNase A under essentially the same conditions were longer than those previously reported<sup>3</sup>, as expected from the frequency dependence of  $T_1$  values<sup>1,5</sup>. The  $T_1$  values observed may reflect the environments of the histidine residues in the structure of RNase A (ref. 29). In view of the known binding of Cu<sup>2+</sup> ion to these residues<sup>30</sup>, however, and the phenomenon reported here for imidazole, such interpretations may not be justified.

The above considerations may not be as important for groups which do not bind metal ions. Nor would such a large effect be expected for the  $T_1$  values of carbon nuclei observed by <sup>13</sup>C NMR. Other studies, however, have indicated the sensitivity of <sup>13</sup>C resonances to paramagnetic metal ions<sup>31</sup>. In view of the above, interpretations of regional unfolding on denaturation of proteins from relative values of  $T_1$  (refs 3, 7, 8) must be viewed with some caution, and may also reflect local changes of ion binding on changing solution conditions and conformation. Addition of EDTA to protein solutions may not simply solve the problem of such measurements since EDTA binds to some proteins<sup>32</sup>. For many other proteins, there is a requirement for a metal ion<sup>3,33</sup>, which although not itself paramagnetic may be accompanied by sufficient paramagnetic metal ion impurity to affect the  $T_1$  values significantly.

We thank Edwin Becker, Philip T. Pitner and Regitze Vold for helpful discussions, Rolf Tschuden for implementing the Varian  $T_1$  program, Charles Mueller for atomic absorption measurements, and Hideo Kon for help in running ESR spectra. R. W. thanks the National Research Council of Canada for financial support.

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Received December 31, 1973; revised March 27, 1974.

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## Detection of metabolic carcinogen intermediates in urine of carcinogen-fed rats by means of bacterial mutagenesis

SEVERAL developments suggest the possibility of a new means of assessing the exposure of human populations to chemical carcinogens. Carcinogens such as 2-acetylaminofluorene (AAF) and *p*-dimethylaminoazobenzene (DAB)

have been shown<sup>1-4</sup> not to be carcinogenically active agents themselves, but to be converted into such agents by metabolic processes in the target organs (in these instances, liver). These studies also show that actively carcinogenic metabolites (as well as other metabolic products which are not carcinogenic) may be excreted in the urine, apparently as glucuronides<sup>4,5</sup>. Similar results have been obtained with human subjects ingesting aflatoxin B<sub>1</sub> from contaminated peanut butter<sup>6</sup>.

Miller *et al.* showed that active AAF-intermediates are mutagenic in a test system involving *in vitro* interactions with transforming DNA followed by inoculation of the latter into a suitable strain of *B. subtilis*<sup>7,8</sup>. More recently Ames *et al.* have found that 18 of 20 chemical carcinogens that were studied are highly mutagenic toward *Salmonella typhimurium* test strains if they are incubated during exposure of the bacteria in a partially purified liver homogenate (a 9,000g supernatant)<sup>9</sup>. These observations considerably reduce the disparity between carcinogenicity and mutagenicity of organic compounds, which has until now discouraged the use of mutagenesis as a test for carcinogenicity. The technique developed by Ames *et al.* is simple, rapid, and sensitive (capable of detecting less than 1 µg of AAF) and hence well adapted to screening of environmental samples. We have investigated whether, in rats exposed to a carcinogen, metabolic intermediates of the latter can be detected in the urine by means of their mutagenic activity in the *Salmonella* test system.

Control male Holtzman rats were maintained on a balanced semisynthetic diet (see ref. 9 for details); experimental groups were maintained on the same diet supplemented with 0.67 mg g<sup>-1</sup> of AAF or 0.6 mg g<sup>-1</sup> of DAB. The animals were supplied with distilled drinking water *ad libitum*. At one week intervals after the onset of feeding, two rats from each group were placed in separate collecting

Table 1 Mutagenic substances in urine of rats fed control and carcinogen-containing diets

| Diet<br>(no. of weeks) | Sample<br>Type | Treatment                           |                                         |                      | No. of colonies*          |                                  | Equivalent<br>conc. of<br>AAF in<br>urine<br>µg ml <sup>-1</sup> † |
|------------------------|----------------|-------------------------------------|-----------------------------------------|----------------------|---------------------------|----------------------------------|--------------------------------------------------------------------|
|                        |                | Equivalent<br>vol. of<br>urine (ml) | β-glucuronidase<br>and<br>Taka-diastase | Liver<br>preparation | Total<br>no. per<br>plate | No. per<br>plate per ml<br>urine |                                                                    |
| Control (1)            | 3 × conc.‡     | 0.9                                 | —                                       | —                    | 16                        | —                                | —                                                                  |
| Control (1)            | Direct         | 0.3                                 | —                                       | +                    | 27                        | —                                | —                                                                  |
| Control (2)            | 3 × conc.      | 0.9                                 | —                                       | —                    | 14                        | —                                | —                                                                  |
| Control (2)            | 3 × conc.      | 0.9                                 | —                                       | +                    | 33                        | —                                | —                                                                  |
| Control (3)            | Direct         | 0.6                                 | —                                       | —                    | 18                        | —                                | —                                                                  |
| Control (3)            | Direct         | 0.6                                 | —                                       | +                    | 25                        | —                                | —                                                                  |
| Control (3)            | Ether extract§ | 0.9                                 | +                                       | —                    | 12                        | —                                | —                                                                  |
| Control (3)            | Ether extract  | 0.9                                 | +                                       | +                    | 22                        | —                                | —                                                                  |
| Control (4)            | 3 × conc.      | 0.9                                 | —                                       | —                    | 18                        | —                                | —                                                                  |
| Control (4)            | 3 × conc.      | 0.9                                 | —                                       | +                    | 35                        | —                                | —                                                                  |
| AAF (1)                | 3 × conc.      | 0.9                                 | —                                       | —                    | 63                        | 70                               | 0.25                                                               |
| AAF (1)                | Direct         | 0.3                                 | —                                       | +                    | 341                       | 1,137                            | 5.2                                                                |
| AAF (2)                | Direct         | 0.6                                 | —                                       | —                    | 56                        | 93                               | 0.35                                                               |
| AAF (2)                | Direct         | 0.6                                 | —                                       | +                    | 1,947                     | 3,245                            | 16.0                                                               |
| AAF (2)                | Ether extract  | 0.9                                 | +                                       | —                    | 175                       | 194                              | 1.0                                                                |
| AAF (2)                | Ether extract  | 0.9                                 | +                                       | +                    | 3,672                     | 4,060                            | 20.2                                                               |
| AAF (3)                | Direct         | 0.6                                 | —                                       | —                    | 153                       | 255                              | 1.1                                                                |
| AAF (3)                | Direct         | 0.6                                 | —                                       | +                    | 2,292                     | 3,820                            | 18.9                                                               |
| AAF (3)                | Ether extract  | 0.9                                 | +                                       | —                    | 207                       | 230                              | 1.1                                                                |
| AAF (3)                | Ether extract  | 0.9                                 | +                                       | +                    | 6,348                     | 7,053                            | 35.1                                                               |
| AAF (4)                | 3 × conc.      | 0.9                                 | —                                       | —                    | 46                        | 51                               | 0.16                                                               |
| AAF (4)                | 3 × conc.      | 0.9                                 | —                                       | +                    | 1,842                     | 2,047                            | 10.1                                                               |
| DAB (4)                | Direct         | 0.3                                 | —                                       | —                    | 15                        | —                                | —                                                                  |
| DAB (4)                | Direct         | 0.3                                 | —                                       | +                    | 28                        | —                                | —                                                                  |
| DAB (4)                | Ether extract  | 0.9                                 | +                                       | —                    | 26                        | —                                | —                                                                  |
| DAB (4)                | Ether extract  | 0.9                                 | +                                       | +                    | 479                       | 532                              | —                                                                  |

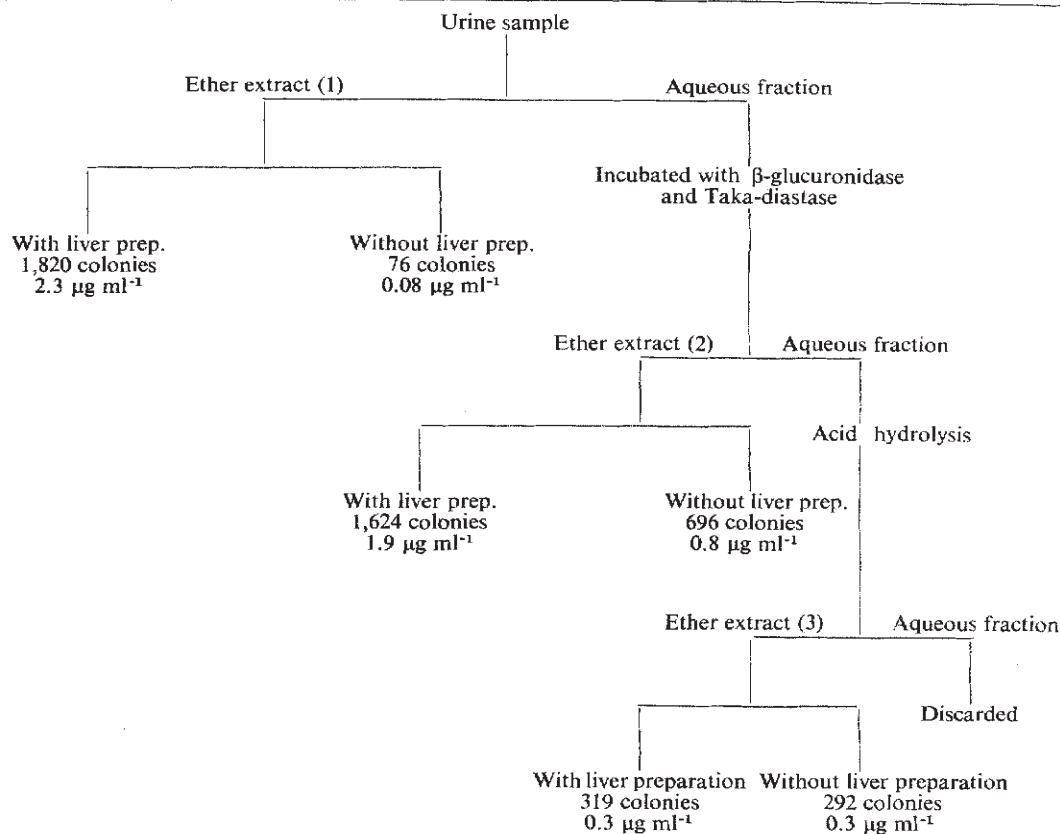
\* Number of revertant colonies produced per plate by *Salmonella typhimurium* test strain TA 1538.

† These values are approximated from the calibration experiment, on the basis that 1 µg of AAF produces about 200 colonies. Corrections have been made for the number of colonies produced by urine samples from rats on the control diet.

‡ Urine concentrates were prepared by evaporating the urine to one-third the original volume.

§ In these instances the urine samples, after incubation with β-glucuronidase and Taka-diastase at pH 7, were extracted with ether. The ether extracts were evaporated to dryness and taken up in one-third the original volume (of urine) of DMSO and aliquots were tested against the bacteria.

Table 2 Analysis of mutagenic constituents of urine



21 ml of urine, adjusted to pH 7 and diluted to 75 ml with water was mixed with 60 ml of 0.1 M pH 7 phosphate buffer and extracted four times with 50 ml aliquots of ether (1). The aqueous phase, freed of ether, was incubated for 16 h at 37° C with 3,200 units each of bovine  $\beta$ -glucuronidase and Taka-diastase (Sigma). After cooling it was extracted four times with 50 ml aliquots of ether (2). The aqueous phase was adjusted to pH 1, refluxed 15 min, neutralised, and extracted four times with 50 ml aliquots of ether (3). The ether extracts (1), (2), and (3) were evaporated and residues dissolved in 2.6, 2.5 and 2.0 ml of DMSO respectively. 0.5 ml aliquots were used for each assay, which represented 4, 4.2 and 5.25 ml of urine for ether extracts 1, 2 and 3 respectively. The procedure is similar to that described in ref. 4. Actual colony counts per plate (test strain TA 1538) are given. Concentrations of mutagens are approximated as described in Table 1.

cages and urine samples were collected for 24 h. The samples were frozen until analysed, and sterilised by Millipore filtration before use.

Tests for mutagenicity were carried out according to the procedures developed by Ames *et al.*<sup>8</sup>, using a strain of histidine-negative *S. typhimurium* (TA 1538) kindly supplied by Dr Ames. In this procedure, the sample to be tested is mixed with aliquots of a 9,000g supernatant prepared from a homogenate of normal rat liver, and with the bacterial inoculum incubated on an agar nutrient medium lacking histidine. Colonies that are formed in this medium represent reverse mutations of the original histidine-negative genome to histidine-positive. Counts of revertant colonies were made after 48 h of incubation at 37° C. All tests were made in duplicate; colony counts are reported as averages of the two values.

First we carried out a calibration experiment to determine the number of mutant colonies of *S. typhimurium* TA 1538 produced when a series of known amounts of AAF were incubated with the 9,000g supernatant. The results showed a linear response in the test range (0–50  $\mu$ g of AAF, that is 0–0.2 mM concentrations of AAF in the final test solution) yielding about 200 colonies per  $\mu$ g of AAF with a blank value of 20 colonies in the absence of added AAF.

Table 1 shows that when rats are maintained on a normal diet for a 4 week period, weekly tests of urine samples show no significant variation in the number of revertant colonies produced either in the presence or absence of the liver preparation. AAF-fed rats excrete in their urine considerable concentrations of mutagenic substances. In the absence of liver preparation, the urine samples obtained

in successive weeks, yielded respectively, 70, 93, 255 and 51 colonies per ml urine. These values are sufficiently above the control value (16 colonies per plate) to suggest the presence of intrinsically mutagenic substances in these urine samples. The evidence relative to the presence of activatable mutagens (that is those activated by exposure to the liver preparation) is unequivocal: in the presence of the liver preparation between 1,137 and 3,820 colonies per ml of urine are observed.

Also shown in Table 1 are the results of tests in which the urine samples were incubated with a mixture of  $\beta$ -glucuronidase and Taka-diastase. The incubation mixture was then extracted with ether, the extract evaporated to dryness, taken up in DMSO (dimethylsulphoxide) and tested in the usual way (see Table 2 for details of this procedure). As shown in Table 1, this procedure considerably increases the mutagenic activity of the samples, both in the presence and absence of the liver preparation. These data indicate that part of both the inherently active intermediates (that is, those which do not require activation by the liver preparation) and of carcinogen intermediates that become active only on treatment with the liver preparation occur in the urine as glucuronides.

DAB was one of the two carcinogens among the 20 tested by Ames *et al.*, which failed to yield mutagenic activity on incubation with the liver preparation. We also found (Table 1) that urine samples from DAB-fed rats tested directly by the Ames method show no mutagenic activity. But when tested according to the procedure (described above) for releasing intermediates from their conjugated forms, significant mutagenic activity is observed. Tested in this way



urine from DAB-fed rats produced 532 revertant colonies per ml of urine, in the presence of the 9,000g supernatant. Thus, DAB-fed rats excrete in their urine glucuronides of intermediates of the carcinogen that are mutagenically active in the presence of the liver preparation, following removal of the sugar residue.

By means of suitable extraction and incubation procedures, similar to those used by Weisburger *et al.*<sup>4</sup>, it is possible to estimate the contribution of the several constituents to the overall mutagenicity of urine samples. The results of such an experiment, using urine from rats maintained on the AAF diet for 1 week, are shown in Table 2. In this experiment the following constituents were found in the urine sample, expressed in amounts equivalent to AAF: (1) activatable, unconjugated mutagen (probably AAF of faecal origin), 2.3  $\mu\text{g ml}^{-1}$ ; (2) intrinsically active, unconjugated intermediates, 0.08  $\mu\text{g ml}^{-1}$  (an amount of marginal significance in this assay); (3) glucuronides of intrinsically active intermediates, 0.8  $\mu\text{g ml}^{-1}$ ; (4) glucuronides of activatable intermediates, 1.1  $\mu\text{g ml}^{-1}$  (1.9–0.8); (5) acid-hydrolysable esters of intrinsically active intermediates, 0.3  $\mu\text{g ml}^{-1}$ . Thus of a total about 4.5  $\mu\text{g ml}^{-1}$  of carcinogen-derived material, 2.2  $\mu\text{g ml}^{-1}$  is certainly of urinary origin and represents both inherently active and activatable intermediates of AAF. Since the mutagenic activity of the urine of DAB-fed rats becomes evident only after treatment with  $\beta$ -glucuronidase and Taka-diastase all of this activity must be due to glucuronides of urinary origin.

Thus metabolic intermediates of AAF and DAB can be detected in the urine of rats fed on diets that contain these carcinogens by means of tests based on mutagenicity towards a strain of *Salmonella typhimurium*, while the urine of rats on normal diets yield no significant response in these tests. This procedure should be applicable to the detection of exposure of human populations to carcinogens. Preliminary tests using human urine are in progress.

This work was supported by grants from the National Institutes of Health.

*Note added in proof:* After the completion of this work we learned that similar results have been obtained by Ames and Durston (*Proc. natn. Acad. Sci. U.S.A.*, **71**, 737; 1974).

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Received December 11, 1973; revised March 22, 1974.

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## Primary defect in copper transport underlies mottled mutants in the mouse

THE results presented here show that a primary defect in copper transport underlies the mottled syndrome in the mouse. The X-linked mottled mutants thus offer an excellent system for the study of mammalian copper metabolism. They also provide an animal model of the inherited human copper deficiency, Menkes kinky hair disease<sup>1,2</sup>, which is also X-linked.

A number of different mutant alleles at the mottled locus are known and these are listed, together with their phenotypic effects, in Table 1. In all cases where the male hemizygote survives beyond birth, the resulting mouse shows a severe dilution in hair pigment (Fig. 1), although the individual hairs are pigmented at their tips, together with an altered hair structure<sup>3</sup> and curly whiskers. A persistent neurological disturbance is also apparent, consisting of a mild sustained tremor and general inactivity<sup>4</sup>. The more severe alleles *Mo* and *Mo<sup>up</sup>* die respectively *in utero* and at birth, and extensive skeletal defects have been reported in *Mo<sup>up</sup>* hemizygotes and heterozygotes<sup>5</sup>.

Recent work<sup>4</sup> has demonstrated a severe deficiency in central noradrenaline (NA) levels in *Mo<sup>br</sup>* and *Mo<sup>vbr</sup>* males and this has been traced to a reduced *in vivo* conversion of dopamine to NA by the enzyme dopamine-beta-hydroxylase (DBH)<sup>10</sup>. A deficiency in NA was also found in mice hemizygous for the viable allele *Mo<sup>bio</sup>* (Table 2). But direct *in vitro* estimates of DBH activity in *Mo<sup>br</sup>* and *Mo<sup>bio</sup>* brain tissue (Table 2) showed an elevated activity, in contrast to the *in vivo* situation. The DBH assay used<sup>11</sup> contains exogenous copper to inactivate endogenous inhibitors of the enzyme. Since DBH is a copper-containing enzyme with a cofactor requirement for cupric ion<sup>12</sup>, the difference between the *in vivo* and *in vitro* results may reside in a copper deficiency in mutant brain tissue.

Estimates of liver and brain copper levels (Table 3) confirm the presence of a copper deficiency in *Mo<sup>br</sup>*. The accumulation of copper in the intestinal wall indicates that uptake from the gut lumen is normal but that the general body deficiency arises from a defective transport within the intestinal epithelial or mucosal cells or across the membrane on the serosal aspect of these cells. To test this interpretation, the *in vitro* uptake of isotopic copper (<sup>64</sup>Cu) into intestinal cells from *Mo<sup>br</sup>* males and normal littermates was examined. Segments of intestinal wall were incubated in Krebs-phosphate buffer containing 1 mg ml<sup>-1</sup> bovine serum albumin and 1  $\mu\text{g ml}^{-1}$  <sup>64</sup>CuCl<sub>2</sub>. The results (expressed in ng <sup>64</sup>CuCl<sub>2</sub> per 5 min per g  $\pm$  s.e.m.) at 0°C (normal: 65.9  $\pm$  11.9; *Mo<sup>br</sup>*: 68.5  $\pm$  11.5) and at 37°C (normal: 131.7  $\pm$  27.4; *Mo<sup>br</sup>*: 138.9  $\pm$  27.7) confirm that the initial uptake into the intestinal epithelium is not defective.

Serum copper is largely contained in ceruloplasmin<sup>15</sup>. Ceruloplasmin levels were estimated by the method of

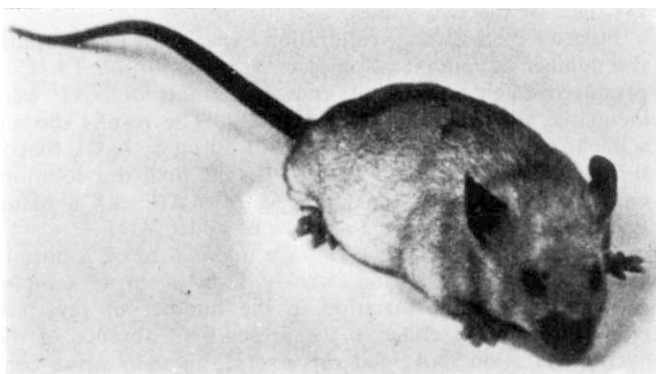


Fig. 1 A rare surviving *Mo<sup>br</sup>* male.

**Table 1** Phenotype characteristics of alleles at the X-linked mottled locus in the mouse

| Allele                                      | Male hemizygote                                                      | Female heterozygote                                                              | Ref  |
|---------------------------------------------|----------------------------------------------------------------------|----------------------------------------------------------------------------------|------|
| Blotchy ( <i>Mo<sup>blo</sup></i> )         | Pale fur, curly whiskers.<br>Viable and fertile.                     | Variegated coat colour, curly whiskers.<br>Viable and fertile.                   | 6    |
| Brindled ( <i>Mo<sup>br</sup></i> )         | Pale fur, curly whiskers.<br>Dies at about 14 d <i>post partum</i> . | Variegated coat colour, curly whiskers.<br>Viable and fertile.                   | 7    |
| Dappled ( <i>Mo<sup>dp</sup></i> )          | Pale fur, curly whiskers, skeletal defects.<br>Dies at birth.        | Variegated coat colour, curly whiskers, skeletal defects.<br>Viable and fertile. | 5    |
| Mottled ( <i>Mo</i> )                       | Lethal <i>in utero</i> .                                             | Variegated coat colour, curly whiskers.<br>Viable and fertile.                   | 7, 9 |
| Viable-brindled ( <i>Mo<sup>vbr</sup></i> ) | Pale fur, curly whiskers.<br>Reduced viability and sterile.          | Variegated coat colour, curly whiskers.<br>Viable and fertile.                   | 8    |

Ravin<sup>13</sup>. The results (normal:  $6.52 \pm 0.60$ ; *Mo<sup>br</sup>*:  $4.33 \pm 0.50$  expressed in  $A_{530}$  units per h per ml serum  $\pm$  s.e.m. show a significant reduction ( $P < 0.01$ ) in ceruloplasmin concentration in mutant mice. The precise function of ceruloplasmin is still obscure but it is unlikely that it represents a copper transport protein since the copper content of ceruloplasmin is not exchangeable *in vivo*<sup>14</sup>. Ceruloplasmin may function in the maintenance of a copper balance<sup>15</sup> and its reduced level in *Mo<sup>br</sup>* supports this interpretation.

The experimental findings are consistent with a deficiency in copper transport and the relationship of a copper deficiency to the different pleiotropic effects of these mutants indicates that it is probably primary. The initial

**Table 2** Brain noradrenaline and *in vitro* activity in *Mo<sup>br</sup>* and *Mo<sup>blo</sup>* ♂♂ and normal (+) littermates

|                         | Age (d) | NA                         | % difference from normal | DBH                  | % difference from normal |
|-------------------------|---------|----------------------------|--------------------------|----------------------|--------------------------|
| +                       | 7       | $1.35 \pm 0.19(6)^\dagger$ |                          | $33.14 \pm 2.39(13)$ |                          |
| <i>Mo<sup>br</sup></i>  | 7       | $0.45 \pm 0.10(6)^\dagger$ | -66.7*                   | $48.81 \pm 2.92(12)$ | +47.3*                   |
| +                       | 28      | $1.96 \pm 0.09(6)$         |                          | $33.02 \pm 3.20(6)$  |                          |
| <i>Mo<sup>blo</sup></i> | 28      | $1.34 \pm 0.06(6)$         | -31.6*                   | $77.63 \pm 5.26(6)$  | +135.1*                  |

DBH activity was assayed by the method of Molinoff *et al.*<sup>11</sup> with the following modifications. Brains were homogenised in 10 volumes of 0.005 M Tris-HCl buffer pH 6.0 containing 0.2% Triton-X100 and assays carried out using tyramine as substrate and a final concentration of  $3.25 \times 10^{-4}$  M  $\text{CuSO}_4$ . NA was assayed by the method of Anton and Sayre<sup>22</sup> as previously modified<sup>10</sup>. DBH activity is expressed as nmol octopamine  $\text{g}^{-1} \text{h}^{-1} \pm$  s.e.m. and NA concentration is expressed in nmol  $\text{g}^{-1} \pm$  s.e.m. Numbers in parentheses refer to number of mice used.

\*Significance at the 1% probability level.

†Data previously published<sup>4</sup>.

effect of a copper deficiency will be on the copper-requiring enzymes and the reduction in the *in vivo* activity of DBH has already been described. The elevated *in vitro* activity of this enzyme may be a reflection of an increased synthesis of the enzyme protein in response to the deficiency in NA biosynthesis. The neurological symptoms are consistent with a deficiency of NA<sup>10</sup>, although a reduction in cytochrome oxidase, another copper-dependent enzyme, may contribute. The virtual absence of hair pigmentation can also be

**Table 3** Copper levels in brain, liver and intestinal wall from 7–10 d *Mo<sup>br</sup>* ♂♂ and normal (+) littermates

|                 | +                   | <i>Mo<sup>br</sup></i> | % difference from normal |
|-----------------|---------------------|------------------------|--------------------------|
| Brain           | $2.43 \pm 0.27(7)$  | $1.43 \pm 0.24(7)$     | -41.1*                   |
| Liver           | $24.23 \pm 1.98(6)$ | $9.37 \pm 1.14(6)$     | -61.3*                   |
| Intestinal wall | $4.39 \pm 0.87(12)$ | $10.13 \pm 1.84(12)$   | +131.3*                  |

Tissue copper levels were estimated by the spectrophotometric method of Carter<sup>23</sup>. Tissue homogenates were hydrolysed with 2N HCl and precipitated with 22.6% trichloroacetic acid. After centrifugation, 0.5 ml of glacial acetic acid containing 40  $\mu\text{g}$  dicyclopentamethylene thiuram disulphide was added to 1.0 ml of tissue supernatant and absorption determined after 10 min at 420 nm. Appropriate standards and blanks were carried through the procedure. Copper levels are expressed in  $\mu\text{g} \text{g}^{-1} \pm$  s.e.m.

\*Significance at the 1% probability level.

Numbers in parentheses refer to numbers of mice used.

explained by a direct effect of the copper deficiency on the activity of tyrosinase, the first enzyme in the melanin biosynthetic pathway. The pigmented tip of each hair may arise from an initial but rapidly depleted copper store in the hair follicle.

Aortic aneurism arising from the fragmentation of the internal elastic lamina of the arterial wall has been reported in a number of mottled alleles<sup>16</sup> and it is significant that the formation of cross linkages between lysine residues in the synthesis of elastin and collagen is a copper-dependent process<sup>17</sup>. Recent results<sup>19</sup> indicate a reduced elastin cross linking in mottled mutants and the lysyl oxidase identified in chick bone collagen<sup>18</sup> seems a likely candidate for the action of the copper deficiency. The skeletal defects may similarly arise from a defective synthesis of bone collagen. The formation of disulphide bonds in keratin is also copper dependent, suggesting defective keratin synthesis as the basis of the curly whiskers and altered hair structure.

Copper deficiency has been shown<sup>2</sup> to underlie the human X-linked progressive brain disease, Menkes kinky hair disease<sup>1</sup>. The phenotypic effects of this mutant include abnormal white hair, altered hair structure<sup>1</sup>, and arterial disease<sup>2</sup>. Copper levels are depressed in liver and serum, and the accumulation of copper in the intestinal epithelium<sup>21</sup> directly parallels that in the mottled syndrome in mice. Catecholamine biosynthesis has not been examined although the reported hypothermia<sup>20</sup> indicates that a deficiency in NA may be present.

Phenotypic variegation arising from random X inactivation<sup>24</sup> in a female heterozygote for an X-linked coat colour mutant depends on the cell autonomy of the mutant defect. The variegated appearance of the mottled heterozygote would therefore argue for the presence of the copper transport defect in the follicle and pigment cells of the skin, and possibly elsewhere. Copper-deficient cells would be expected to be at a selective disadvantage in competition with their normal counterparts, leading to a progressive dilution of mutant cells during development. The progressive darkening of *Mo<sup>br</sup>* heterozygotes with age<sup>3</sup> supports this conclusion.

I thank Drs J. Axelrod and R. Ciaranello of the National Institute of Mental Health, Bethesda, Maryland, for a sample of purified phenylethanolamine-N-methyl-transferase for use in the DBH assay, and Miss K. McGillveray for technical assistance. The work was supported by a Project Grant from the Medical Research Council.

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Received February 8; revised March 25, 1974.

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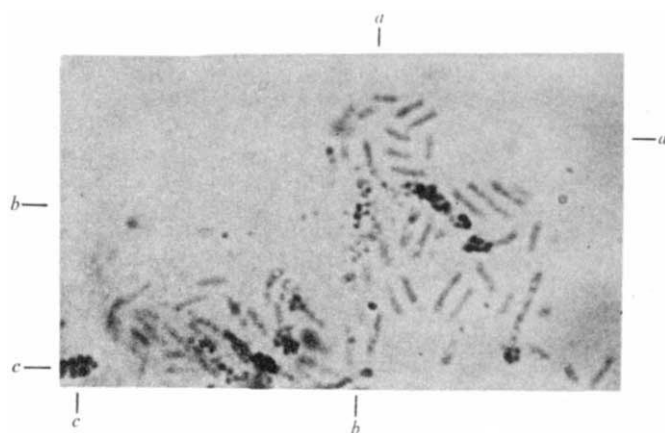
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**Fig. 1** Autoradiograph of *M. leprae* labelled with radioactive DOPA ( $5 \mu\text{Ci ml}^{-1}$  for 48 h). Patient in initial stages of treatment. Light staining for acid-fast bacilli to show contrast with dense silver grains. *a*, Unlabelled bacilli; *b*, separate bacilli showing one to three grains; *c*, small groups of densely labelled bacilli.

## Uptake of radioactive DOPA by *M. leprae*

THE inability to culture *Mycobacterium leprae* *in vitro* has been a great obstacle to the progress of research in leprosy. This obstacle has been in part overcome by the introduction of the morphological index for the assessment of the viability of *M. leprae*, although the index is relatively crude. Biochemical studies<sup>1,2</sup> have revealed the specificity of the enzyme *o*-diphenoloxidase in relation to *M. leprae* as opposed to other bacteria included in the genus of mycobacteria. *o*-Diphenoloxidase oxidises 3,4-dihydroxyphenylalanine (DOPA) to quinone.

It seemed possible that DOPA might be a metabolite of the living bacilli and that radioactive atoms within the molecule would become incorporated into macromolecules within the living organism. We have shown this to be the case by high resolution autoradiography. Bacterial suspensions were prepared by the homogenisation of biopsies from untreated patients without disruption of bacilli and from patients in early stages of treatment, with a relatively high morphological index. Drops of suspension were added to a smear of warm agar on coverslips and incubated for various periods up to 48 h in the presence of  $5 \mu\text{Ci ml}^{-1}$  of  $^3\text{H}$ -DOPA. The cultures were carefully washed and fixed in formol saline and then examined by autoradiography with Ilford K5 Emulsion as a thin film.

The high resolution obtained made it possible to observe the grains on individual bacilli (Fig. 1). A number of bacilli were clearly labelled after 48 h and some were intensely labelled; granular bacilli were unlabelled. The heavily labelled bacilli were often seen as a small centre of compact organisms within a large group. These clearly labelled bacilli might originate from centres of active growth within the population. In a parallel test with  $^3\text{H}$ -thymidine a few of the bacilli in the aggregates showed grains. Labelled bacilli were also observed after uptake by mouse peritoneal macrophages in culture. Drutz and Cline<sup>3</sup> have observed labelling of some globi in human peripheral blood monocytes using thymidine. The high resolution autoradiography combined with the use of DOPA shown here enables the metabolic activity of individual bacilli to be assessed.

By combining the use of labelled DOPA and labelled thymidine it therefore becomes possible to give, in addition to the morphological index, an assessment of metabolic activity (from DOPA labelling) and of rate of multiplication (from thymidine labelling) for individual bacilli of *M. leprae* in a given population. Such measurements can be made on biopsy specimens from new patients and also during treatment of patients as described above. It should also be possible to use the test for short term bacteriological investigations in the laboratory (somewhat similar to those used in the study of effects on metabolism and growth with organisms which grow well *in vitro*) for example to test the effects on metabolism and growth of potential chemotherapeutic agents, of immune serum and of lymphocytes. A more rapid method than autoradiography for assessing uptake of labelled metabolites is to use scintillation counting for bulk suspensions. Preliminary experiments measuring uptake of  $^3\text{H}$ -thymidine by *M. leprae* by this method have also led to promising results.

These methods do not obviate the need for the use of mouse footpad experiments to assess the growth potential of *M. leprae* *in vivo*<sup>4</sup>, particularly the use of immunologically deprived animals<sup>5</sup>. The most useful experimental approach in cell studies is generally to carry out parallel *in vivo* and *in vitro* investigations. The rapidity with which results can be obtained by the method described makes it attractive for a number of laboratory investigations.

We thank Mr Harold Das, Leprosy Home, Poladpur, for providing biopsy specimens from leprosy patients, and Drs Kamal Ranadive and C. V. Bapat for discussions. We thank Drs R. Tchao and D. Easty for advice in preparing the autoradiographs. This study was supported by the World Health Organisation and the Lady Tata Memorial Trust. E. J. A. was a visitor to the Department of Plastic Surgery.

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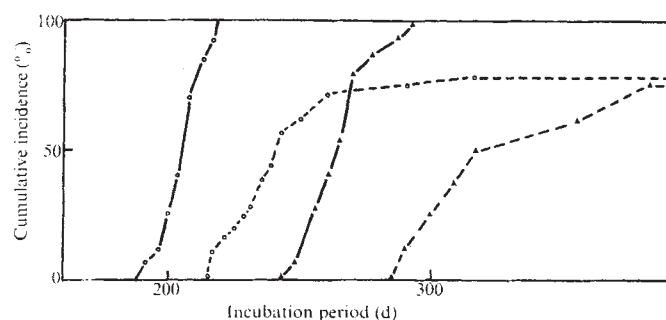
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## Reduced susceptibility to scrapie in mice after steroid administration

ANIMALS deficient in lymphoreticular function either by age, genotype or immunosuppression are more susceptible to infection by most known pathogens mainly on account of their depressed immunological responses. An intact lymphoreticular system (LRS), however, can itself contribute to disease by various mechanisms including; providing sites for multiplication<sup>1</sup>, producing infectious complexes<sup>2</sup> and precipitating autoaggressive accompaniments and sequelae<sup>3</sup>. It is possible that a special class of this paradoxical relationship is exemplified by the slow encephalopathies of which the best understood is scrapie, others being kuru, Creutzfeldt-Jakob disease and mink encephalopathy. In these diseases of the central nervous system, infection normally occurs by some peripheral route and there always seems to be an initial, probably obligatory, replicative phase in the LRS before gaining access to the target organ<sup>4,5</sup>. In spite of this involvement of the LRS, and indicating some very special relationship as its basis, there is a complete absence of conventional immunological responses. No scrapie-specific antibodies<sup>6</sup> or interferons<sup>7</sup> have been found, and this is not due to any generalised depression of immune reactions<sup>8</sup>. Immunosuppressive techniques, which severely impair the functions of the B and T-cell elements of the LRS, do not affect the pathogenesis of peripherally injected scrapie<sup>9,10</sup>. Also congruent with an absence of immune responses is the non-inflammatory nature of the brain lesions even in the later stages of the disease<sup>11,12</sup>.

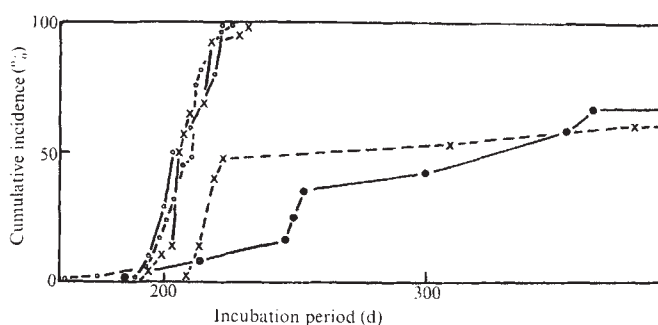
A number of findings, however, can be construed as indicating some positive relation between LRS function and the patho-



**Fig. 1** Cumulative incubation periods for VL mice injected intraperitoneally (i.p.) as adults (21-42 d old) with 10% brain homogenate (500g, 10 min supernate) of 79A-strain scrapie agent (○) or 22C agent (▲); with (— — —) or without (—) the following high-dose prednisone acetate regime. Three days before the scrapie injection, 0.1 ml of a suspension of PA (250 mg kg<sup>-1</sup>) in 2% Tween 20 in saline was injected subcutaneously (s.c.) over the shoulder, and then, beginning the day after the scrapie injection, 21 daily injections of PA suspension (15 mg kg<sup>-1</sup>) were given s.c. as a maintenance dose. The controls received Tween 20 in saline only. The 0.02 ml injections of scrapie in saline contained approximately 1,000 i.p. LD<sub>50</sub> infectious units.

genesis of scrapie agents. Apart from the early high titres of agent in these tissues<sup>13</sup>, there is an increased incubation period in splenectomised<sup>14</sup> and spleenless mutant mice<sup>15</sup>; the unexpected finding of decreased susceptibility to extraneurally injected scrapie in newborn mice<sup>16</sup>; the demonstration that lymphocytes can become sensitised to scrapie-damaged tissue<sup>17</sup>; and the decreased levels of circulating polymorphonuclear neutrophils soon after scrapie infection<sup>18,19</sup>.

In the light of these findings it is interesting to report that the pathogenesis of scrapie in young mice can be profoundly modified by early injections of the anti-inflammatory steroid prednisone acetate (PA). Injections of PA in physiologically high doses on the days immediately before and after an intraperitoneal (i.p.) injection of scrapie-infected brain homogenate results, unexpectedly, in prolonged incubation periods and a



**Fig. 2** Cumulative incubation periods for VL mice of different ages injected with 79A scrapie agent, together with (— — —) or without (—) a single moderate dose of PA. A similar 79A brain homogenate to that used in Fig. 1 was injected (i.p.) into mice aged 22-46 d (○), 14-20 d (×) or on the day of birth (●) (using due precautions against leakage). Half of the 22-46 and 14-20 d-old animals received a single s.c. injection of PA (50 mg kg<sup>-1</sup>) in 2% Tween 20 in saline 3 d before the scrapie injection. No neonates were given PA treatment.

proportion of long term survivors using an agent dose which is 100% lethal in untreated controls (Figs 1 and 2). As the doses required for the effect rise rapidly with the age of the host, it is possible that the drug operates on the same tissues as those responsible for the reduced susceptibility of neonatal mice to scrapie<sup>16</sup>.

Figure 1 shows the effect on incubation periods of injecting adult VL mice with 79A or 22C scrapie, i.p., with or without a short, intensive course of PA injections at high dose levels during the first 3 weeks of infection. These steroid injections caused prolonged incubation periods, as judged by the onset of clinical disease seven months later, and a proportion (20%) of long term survivors, that is, individuals still alive and healthy more than 200 d after the death of all mice without steroid treatment. (In earlier pilot experiments some of these animals lived more than 700 d without contracting the disease although there were also some very late cases).

Figure 2 shows the effect of age and of a much lower dose of PA on the incubation period of 79A agent in VL mice of different ages between birth and 6 weeks. The single, previous injection of PA had no effect on the pathogenesis of the older animals (22-46 d at the time of scrapie injection), but it prolonged the incubation period of the younger age group (14-20 d) and caused 40% long term survivors. The cumulative incidence curve for this latter group is very similar to that for the neonatal mice which were injected at the same time and with the same dose of agent, but without a PA injection.

In spite of the fact that PA shares a number of immunosuppressive effects with thymectomy<sup>9</sup>, and cyclophosphamide<sup>10</sup> and other drugs<sup>20</sup> it seems unlikely that these shared effects, particularly on B and T cells, are the basis of its influence



on scrapie incubation. It is not yet possible, however, to identify the relevant tissues or functions, which may indeed involve some that are not yet understood biologically<sup>21</sup>. Interim results from experiments to identify the relevant effects of PA show that: the kind of PA regime described seemed to have no effect on agent injected intracerebrally; there was no agent in the spleen of PA-treated mice surviving without signs to 340 d after scrapie injection; neonatal injections of PA did not affect scrapie susceptibility permanently (that is, by a developmental effect); and a variety of other PA regimes have been shown to be ineffective in modifying scrapie incubation periods (initial high dose without the maintenance course in adult mice); or high doses of PA either 6 weeks after i.p. injection of agent, or after the onset of clinical signs.

Before attempting to formulate detailed models based on these observations it will be necessary to be more specific about which tissues and cell types are involved. This will entail the use of other steroids and procedures, with different and more selective actions on various cell populations, together with a study of the dynamics of titre rise in those populations. We anticipate that macrophages, reticular cells, capillary sheath cells, polymorphonuclear neutrophils, mast cells, relatively steroid-resistant lymphocytes, various stem cells and sympathetic nerve terminals will yield the cell types involved. Sympathetic nerve terminals are not an obvious choice but they have several attractive features and have been discussed in connection with mink encephalopathy<sup>22</sup>. As a nervous, non-lymphoid site of replication they would provide an explanation for the moderately high titres of agent found in organs such as the lungs, salivary glands and adrenals, and would permit the hypothesis that replication occurs in only one cell type instead of at least two. This hypothesis is more satisfactory in view of the fact that the onset of replication in both the periphery and the brain is controlled by the same gene<sup>23</sup>, furthermore, the metabolism of sympathetic neurones can be modified by high doses of glucocorticosteroids<sup>24</sup>. This possibility would not, of course, exclude a role for lymphoid elements in the uptake, accumulation and transportation of agent.

These findings carry the important experimental implication that attempts to encourage intraspecific passage of these 'slow viruses' by immunosuppressing the prospective hosts may be self-defeating<sup>25</sup>. As scrapie and other slow encephalopathies must infect by a peripheral route in the natural disease, a knowledge of the details of the early steps in their pathogenesis—especially as these include some natural mode of inactivation—could be relevant for devising prophylactic treatments. Further investigation of this steroid effect could help to identify some of these early steps in infection and agent inactivation, and help to explain why the lymphoid system appears to act as a 'Trojan horse' instead of a defence mechanism in these diseases.

We thank the Association of Scottish Branches of the Multiple Sclerosis Society for financial help.

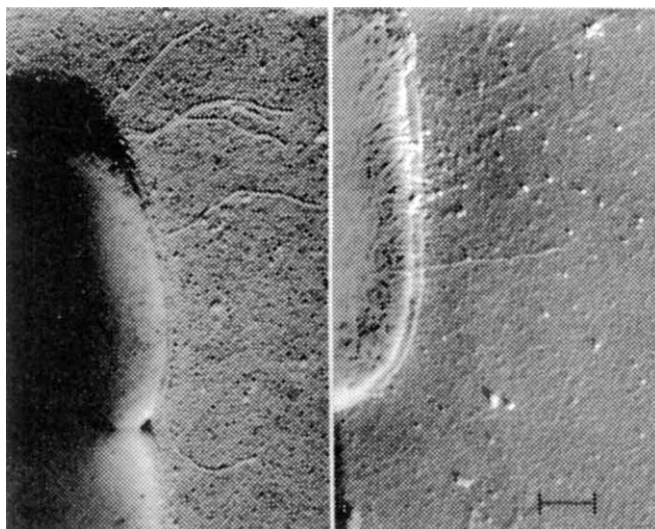
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## Absence of a pilus receptor for filamentous phage IKE

PLASMID-SPECIFIC filamentous bacteriophages have been known to attach to the tips of certain pili present on the surface of their *Escherichia coli* or *Salmonella typhimurium* hosts; the synthesis of these appendages is determined by specific plasmids which reside in the host cell<sup>1–4</sup>. As well as providing receptor sites for such phages, these pili have been found to be essential for the conjugal transfer of the plasmids<sup>5–7</sup> and some hypotheses of



**Fig. 1** The attachment of IKE particles to the surface of *E. coli* RM98<sup>+</sup> cells ( $\times 81,600$ ). The scale represents 100 nm.

Received January 29, 1974.

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**Table 1** Adsorption of M13 and IKE to formalin killed F42<sup>+</sup> and RM98<sup>+</sup> cells

| Experiment | Plasmid | Phage | MOI    | Treatment of cells | Phage input (c.p.m. ml <sup>-1</sup> phage/cell mixture) | C.p.m. ml <sup>-1</sup> recovered in phage/cell complexes† | Unadsorbed phage (% of input) |
|------------|---------|-------|--------|--------------------|----------------------------------------------------------|------------------------------------------------------------|-------------------------------|
| A*         | F42     | M13   | 20     | Unblended          | 13,500                                                   | 393                                                        |                               |
|            | F42     | M13   | 20     | Blended            |                                                          | 255                                                        |                               |
| B*         | F42     | M13   | 20     | Unblended          | 7,000                                                    | 123                                                        |                               |
|            | F42     | M13   | 20     | Blended            |                                                          | 95                                                         |                               |
| C*         | RM98    | IKe   | 20     | Unblended          | 19,700                                                   | 226                                                        |                               |
|            | RM98    | IKe   | 20     | Blended            |                                                          | 801                                                        |                               |
| D*         | RM98    | IKe   | 20     | Unblended          | 16,900                                                   | 234                                                        |                               |
|            | RM98    | IKe   | 20     | Blended            |                                                          | 676                                                        |                               |
| E‡         | F42     | M13   | 0.0007 | Unblended          |                                                          |                                                            | 77                            |
|            | F42     | M13   | 0.0007 | Blended            |                                                          |                                                            | 99                            |
| F‡         | RM98    | IKe   | 0.0007 | Unblended          |                                                          |                                                            | 83                            |
|            | RM98    | IKe   | 0.0007 | Blended            |                                                          |                                                            | 68                            |

\* Phages were labelled with <sup>3</sup>H-thymidine. Phage/cell mixtures were layered on L broth containing 10% sucrose and centrifuged at 2,800 r.p.m. at 4° C for 25 min (ref. 12). The pellets were resuspended in L broth and 1 ml aliquots counted after mixing with 10 ml aquasol (New England Nuclear) in a Beckman LS-250 liquid scintillation system.

† The figures in this column have been corrected for background counts (30–40 c.p.m. ml<sup>-1</sup>) obtained from counting 1 ml of the uninfected plasmid-bearing culture in 10 ml aquasol.

‡ Formalin-killed cells were washed free of formalin on sterile Millipore filters before resuspending in L broth and infecting with phage. Phage/cell mixtures were centrifuged following incubation for 10 min at 37° C and the supernatant assayed for unadsorbed phage on Hfr or RM98<sup>+</sup> indicator strains.

conjugal transfer consider that they are directly involved in the transfer process<sup>6,8</sup>. Previously, we have described a filamentous phage IKE, specific for the R factor RM98 and other R factors of the N compatibility group<sup>9</sup>. Here we describe evidence which indicates the direct attachment of IKE to the surface of its host which lacks an observable pilus-like appendage.

An *E. coli* F<sup>-</sup> auxotroph, strain JE2571, unable to synthesise common pili or flagella was used as a host for RM98. This host was capable of synthesising F- and I-type pili following the introduction into it of the plasmids F42 and a derepressed mutant of Col I transferred from *S. typhimurium* LT2 ColI<sup>drd</sup>. Strains bearing F42 or RM98 were grown to logarithmic phase in L broth<sup>10</sup>; the ColI<sup>+</sup> strain was cultivated in trypticase soy broth. These conditions yielded cells which always showed maximum adsorption of the respective phages. Drops of these cultures were placed on carbon-coated, formvar-supported 400 mesh copper grids. Dried grids were washed by touching the formvar surface of the grid to four drops of sterile distilled water. Shadowing was done at an angle of 11° with platinum-palladium or platinum-carbon in an Edwards High Vacuum Evaporator. The grids were examined in a Philips 300 electron microscope used at an accelerating voltage of 60 kV, at a main screen magnification of 14,000–35,000. The F42<sup>+</sup> and ColI<sup>+</sup> cultures showed their respective pili which were either surface attached or free. The RM98<sup>+</sup> strain showed neither surface attached nor free pili when we examined 10 grids prepared on three different occasions. We examined 1,300 isolated cells whose surfaces were clearly visible. At the magnification used, linear structures having a diameter of 5 nm or more should have been detected. Neither were any protrusions suggestive of very short pili observed.

In a second and independent approach, we assumed that if pilus-type appendages attached to the surface were present, they would be mechanically removable by agitating the cells. This treatment is known to detach F pili from the surface of F<sup>+</sup> cells<sup>7,11</sup>.

L broth cultures of F42<sup>+</sup> or RM98<sup>+</sup> cells grown at 37° C to the logarithmic phase were chilled and treated with formalin (3.7% v/v) for 5 min. No viable cells could be recovered following this treatment which has been used to prevent the outgrowth of F pili following blending<sup>11</sup>. An aliquot of the formalin treated culture (40 ml) was blended at 0° C for 2 min at 10,000 r.p.m. in a 250 ml polypropylene bottle of a Sorvall Omnimixer to yield the 'blended' preparation (Table 1). Each cell prepara-

tion was infected with purified phage M13 or IKE; the phages were either labelled with <sup>3</sup>H-thymidine or unlabelled. Phage/cell mixtures were incubated at 37° C rather than at 0° C because IKE does not adsorb at the lower temperature.

The results in Table 1 indicate a reduction in the ability of blended F42<sup>+</sup> cells to adsorb M13. At high multiplicities of infection this reduction was 23–35% (Table 1, Experiments A and B) and 96% at low multiplicities of infection (Table 1, Experiment E) relative to unblended controls. Blended RM98<sup>+</sup> cells showed a two to three-fold increase in IKE adsorption; this was observed at high as well as low multiplicities of infec-

**Table 2** Complex formation between IKE and RM98<sup>+</sup> cells at 37° C in L broth

| Incubation (min) | % cells with attached IKE* | Numbers of IKE particles attached per cell |      |      |           |
|------------------|----------------------------|--------------------------------------------|------|------|-----------|
|                  |                            | 1                                          | 2    | 3    | 4 or more |
| 0                | 0.4                        | 0.2                                        | 0.2  |      |           |
| 1                | 5.24                       | 4.5                                        | 0.5  | 0.24 |           |
| 2                | 16.9                       | 15.3                                       | 1.6  |      |           |
| 4                | 42.2                       | 28.4                                       | 10.2 | 1.6  | 2.0       |
| 7                | 42.6                       | 26.2                                       | 10.4 | 4.9  | 1.1       |
| 10               | 43.4                       | 24.1                                       | 10.4 | 6.0  | 2.9       |

Phage/cell complexes were permitted to form at 37° C for 0–10 min in L broth and chilled immediately to prevent further adsorption. The complexes were separated from unadsorbed phage as described in the legend to Table 1. Pellets from the sucrose centrifugation were washed with chilled water and used for making grids which were shadowed.

\* Three grids were prepared at each incubation time and at least 150 isolated cells examined per grid.

tion (Table 1, Experiments C, D and F). High speed blending of hosts bearing R factors of the N compatibility group has been reported to be ineffective in removing IKE receptors<sup>13</sup>.

This evidence indicates the absence of a pilus-type receptor for IKE. It was therefore reasonable to expect that the phage would be seen to attach directly to the cell surface on which the receptor was located. This was in fact observed (Fig. 1) when complexes formed after mixing phage and cells were examined either immediately or following incubation of the mixtures at 37° C. The only difference noted was in the numbers of cells which showed attached phage particles (Table 2) but no difference could be seen in the manner of this attachment.

We conclude that a pilus-type receptor is not involved in the attachment of IKE to RM98<sup>+</sup> cells when the latter are grown in conditions which give a maximum specific susceptibility to



infection by IKE. We are now attempting to identify the location and nature of this receptor.

We thank Dr G. H. Haggis and Mr S. Itz for the use of electron microscope facilities at the Canada Department of Agriculture, Ottawa and Dr V. N. Iyer for editorial comments. This research was supported by a predoctoral trainee award to P. B. and an operating grant to R. I. from the Medical Research Council of Canada. *E. Coli* strain JE2571 was provided by Y. Nishimura and *S. typhimurium* LT2 Coll drd by G. G. Meynell.

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Received February 14; revised March 18, 1974.

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## Photophosphorylation of an ADP analogue by spinach chloroplasts

THE mechanism of ATP synthesis during photosynthetic phosphorylation is not yet known. It has been shown, however, that a coupling factor protein is necessary for coupling ATP synthesis to electron transport<sup>1</sup>. An interesting mechanism for photophosphorylation has been proposed<sup>2,3</sup> whereby photosynthetic formation of ATP is brought about by transphosphorylation of two coupling factor-bound ADP molecules to form AMP and ATP. The AMP would then be rephosphorylated in a step dependent on electron transport to form coupling factor-bound ADP which would act as the high energy intermediate (which has been thought to exist) to phosphorylate another ADP molecule to ATP.

We have used  $\alpha,\beta$ -methylene adenosine 5'-diphosphate (AOPCP), an analogue of ADP, to determine whether ADP undergoes transphosphorylation to AMP and ATP during photosynthetic ATP formation. Since the two phosphates in AOPCP are linked by a non-hydrolysable-methylene group<sup>4</sup>, this ADP analogue cannot transfer its  $\beta$  phosphate in a myokinase type of reaction. Any photophosphorylation of AOPCP to  $\alpha,\beta$ -methylene adenosine 5'-triphosphate (AOPCPOP) would therefore mean that photosynthetic formation of ATP is due to direct phosphorylation of ADP. Our results indicate direct phosphorylation of ADP to ATP.

Experiments in which AOPCP was used as substrate for

the photophosphorylation reaction are shown in Table 1. Incubation mixtures containing AOPCP incorporated  $P_i$  into 'organic phosphate'. This reaction was light-dependent since no incorporation was obtained in the dark (experiment 1). The incorporation was not due to the presence of endogenous nucleotides since very little activity was found when AOPCP was omitted (experiment 2). Photosynthetic phosphorylation of AOPCP to AOPCPOP was catalysed at rates of 30 to 50  $\mu$ mol AOPCPOP formed per h per mg chlorophyll. This reaction was six to ten times slower than phosphorylation of ADP. One of the reasons for the slower phosphorylation of AOPCP might be the differences in bond angles and distances of the  $P-CH_2-P$  and  $P-O-P$  groups<sup>5</sup>, and so AOPCP may not fit into the active site for photophosphorylation as well as does ADP. The time course of photophosphorylation of AOPCP showed that the reaction was almost linear for 10 min, at which time more than 0.8  $\mu$ mol of the total of 6  $\mu$ mol of AOPCP in the incubation mixture was esterified with  $^{32}P_i$ .

To show conclusively that AOPCP was phosphorylated to AOPCPOP during photophosphorylation, the nucleotides

**Table 1** Photophosphorylation of AOPCP by a spinach chloroplast preparation.

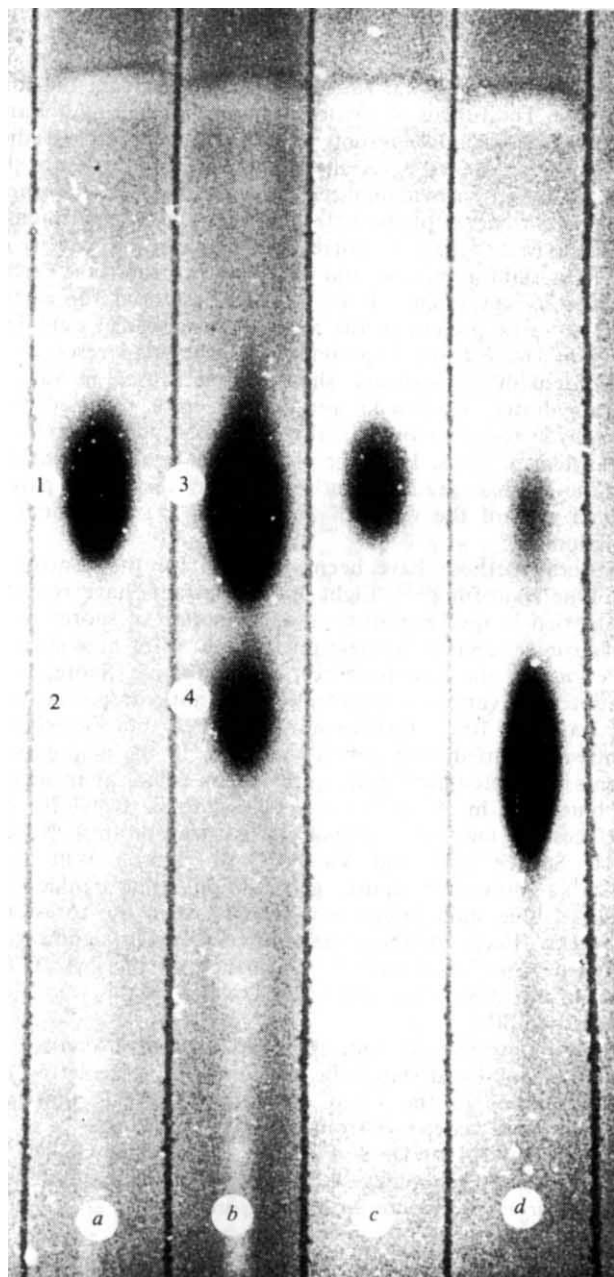
| Nucleotide added | Illumination | $P_i$ esterified<br>( $\mu$ mol per h per mg chlorophyll) |
|------------------|--------------|-----------------------------------------------------------|
| Experiment 1     |              |                                                           |
| AOPCP            | No           | 0                                                         |
| AOPCP            | Yes          | 47                                                        |
| Experiment 2     |              |                                                           |
| None             | Yes          | 0.4                                                       |
| ADP              | Yes          | 299                                                       |
| AOPCP            | Yes          | 31                                                        |

One hundred grams of deveined spinach leaves were homogenised in 300 ml of 0.4 M sucrose containing 40 mM TES-NaOH and 0.2% fatty acid poor bovine serum albumin (Calbiochem) at pH 8 in a Waring blender. The homogenate was filtered through eight layers of cheesecloth and centrifuged for 5 min at 4,000g. The chloroplast pellet was resuspended in a solution containing 45 ml of the homogenisation medium and 15 ml of glycerol. Aliquots (1 ml) of the resulting suspension were stored in sealed vials in liquid nitrogen<sup>6</sup> and were thawed at room temperature immediately before use. Photophosphorylation was measured as described previously<sup>6</sup> using 2 mM ADP (Sigma) or AOPCP (Miles Laboratories) and 0.1 ml of chloroplast suspension (117  $\mu$ g of chlorophyll). Incubation time was 5 min at 40,000 lux of incandescent light, or in the dark, as indicated. Chlorophyll was measured in 80% acetone extracts using the equation of MacKinney<sup>7</sup>.

from denatured incubation mixtures were separated by thin layer chromatography (Fig. 1). The reaction mixture incubated in the dark contained only AOPCP (spot 1), while the light incubated sample contained both AOPCP (spot 3) and AOPCPOP (spot 4). Radioactivity was found only in the AOPCPOP spot indicating that  $^{32}P_i$  reacted with AOPCP to form AOPCPOP during photophosphorylation.

These results indicate that the ADP which serves as substrate in the photophosphorylation reaction is phosphorylated directly to ATP and does not undergo a transphosphorylation to AMP and ATP. Roy and Moudrianakis<sup>2,3</sup>, Forti *et al.*<sup>10</sup> and McPhee and Brody<sup>11</sup>, however, have obtained evidence for a transphosphorylation reaction. Moreover, both chloroplast<sup>2</sup> and mitochondrial<sup>12,13</sup> coupling factors have been found to bind adenosine nucleotides strongly. It is possible therefore, that the isolated chloroplasts contained coupling factor-bound ADP which underwent transphosphorylation to AMP and ATP and that the resultant coupling factor-bound ATP served as the 'high energy intermediate' for the phosphorylation of AOPCP. If this

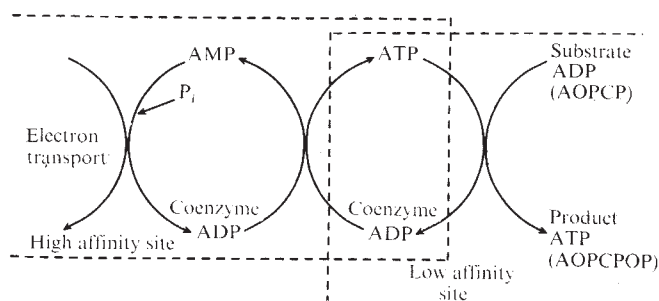
were the case, our results would point to the existence of two separate ADP pools involved in photophosphorylation. One would be the substrate ADP (represented by AOPCP



**Fig. 1** Thin layer chromatography of nucleotide analogues. Chromatography was on cellulose coated Eastman Chroma-gram sheets using the solvent system isobutyric acid: concentrated ammonia: water = 66:1:33 (ref. 4). To remove salts which interfered with the separation, nucleotides from deproteinized incubation mixtures (3.2 ml) were adsorbed on 0.2 g of acid washed Norit charcoal. The charcoal was washed three times with 5 ml  $H_2O$  and nucleotides were eluted with 5 ml of 1%  $NH_3$  in 60% ethanol<sup>9</sup>. The eluates were concentrated and aliquots used for chromatography. The resulting chromatograms were photographed under ultra-violet light with a Wratten 2A filter. Top and bottom correspond to front and start respectively. Chromatographed samples were: a, 10 min dark incubation; b, 10 min incubation at 40,000 lux; c, AOPCP standard; d, AOPCPOP standard. Analogue standards were purchased from Miles Laboratories. Numbered areas were scraped out and radio-activity of the material was counted in the liquid scintillation counter. The activities were: spot 1, 20 c.p.m.; 2, 17 c.p.m.; 3, 28 c.p.m.; 4, 1,278 c.p.m.

in our experiments) which would undergo direct phosphorylation to ATP. The other would be coupling factor-bound ADP which would act as a coenzyme for the coupling factor and which would undergo the transphosphorylation reaction proposed by Roy and Moudrianakis<sup>2,3</sup>. Figure 2 shows the reactions which might occur on the coupling factor; the proposed scheme is in agreement with our results as well as those of Roy and Moudrianakis<sup>2,3</sup>. It assumes that coenzyme ADP which undergoes the myokinase type of reaction stays bound to the coupling factor while the substrate ADP associates with the coupling factor only transiently to accept the phosphate from coupling factor bound ATP produced by the myokinase-like reaction.

It has been observed<sup>12</sup> that the mitochondrial coupling factor contains two binding sites for nucleotides, one with high and one with low affinity for ADP. The low affinity site was identical with the active site for the ATPase reaction catalysed by the coupling factor. The function of



**Fig. 2** Hypothetical scheme of reactions occurring on the coupling factor during photophosphorylation. The high affinity site is assumed to contain coupling factor bound nucleotides while the low affinity site is thought to be the site of phosphorylation of substrate ADP.

the high affinity site is not known. Since the mitochondrial and chloroplast coupling factors are very similar, it is possible that the high affinity binding site is the site of the myokinase-like reaction involving the coupling factor-bound coenzyme ADP, while the low affinity site is the site of phosphorylation of substrate ADP. Reactions at the high affinity site should therefore have the properties ascribed to the formation of the 'high energy intermediate', while reactions at the low affinity site should be identical to those involving the last step in photosynthetic ATP formation.

This research was supported by a grant to S. Z. from the National Research Council of Canada. A.H. is a recipient of the National Research Council of Canada and University of Alberta Killam Postdoctorate Fellowships. We thank Mr Barry Zytaruk for preparing the figures.

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Received January 9, 1974.

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## Sap stain in *Antiaris africana*, an economically important tropical white wood

MANY countries lose thousands of pounds annually through natural staining of valuable wood. Usually the organism which causes staining is the fungus *Botryodiplodia theobromae* Pat. Field and laboratory experiments have shown that *Antiaris africana* becomes stained blue within 4 d of felling<sup>2,3</sup>. The fungus enters the cut surface of the wood through the vessels and within 2 d heavily colonises the surrounding vascentric parenchyma cells. Infection of ray cells occurs through the vascentric parenchyma cells<sup>1</sup>. The fungus has two types of hyphae: some are stout and brown in colour, others are thin and hyaline. The brown hyphae are predominantly found in vessels, tracheids and fibres (Fig. 1), whereas the parenchyma cells are heavily infected by the hyaline hyphae.

It is significant that not all fungi to which cut logs of *A. africana* are exposed gain entrance to the wood through vessels. *Phialophora fastigiata*, a sap stainer with a tendency to behave like a soft-rot fungus enters the wood through the ray parenchyma cells from which it ramifies into the vascentric parenchyma cells. The vessels are infected through

parenchyma cells. Within 14 d of infection, the fungus successfully digests the secondary wall of the fibres, creating cavities in oblique, transverse and longitudinal directions. *Rhinocladia atrovirens*, a sap stainer, and *Chaetomium globosum* infect the wood through the rays and the vascentric parenchyma cells.

Shoots of *A. africana* were exposed to <sup>14</sup>CO<sub>2</sub> for 45 min three times a week for 2 weeks and then cut into small logs. The cut surface of each log was sprayed with spores of *B. theobromae* and the fungus was isolated from the logs after 7 d. The fungus was extracted with boiling ethanol and the ethanol-insoluble residue was wet-combusted<sup>4</sup>. Radioactivity was detected especially in the ethanol extract of the isolated fungus, providing direct evidence that *B. theobromae* uses food reserves of the xylem. There is also experimental evidence that there is no correlation between the severity of the blue-stain infection and the concentration of carbohydrate in the xylem<sup>5</sup>. It is, however, essential for carbohydrate to be present in the xylem for successful establishment of the fungus. Experiments on the food reserves of the xylem of *A. africana*<sup>5</sup> show that it is rich in various carbohydrates. Kozłowski and Keller<sup>6</sup> have reported that carbohydrates are stored in the living parenchyma cells of the xylem of trees. It is, therefore, most probable that the hyaline hyphae predominantly found in the living parenchyma cells of the wood of *A. africana* are nutritional in function.

Several methods have been suggested for the control of sap blue stain fungi<sup>7,8</sup>. Eight antitranspirants have recently been tried in the laboratory on germination of spores of *B. theobromae*, and in the field on the control of blue stain in *A. africana*, and four have been very effective. Spores were mounted in various concentrations of antitranspirants and incubated at 100% relative humidity. Percentage germination was counted over a period of 24 h. In the field experiments conducted during the rainy season when the incidence of blue-stain in *A. africana* is known to be heaviest<sup>2</sup>, cut surfaces of logs were dipped in antitranspirants. Needle Fast, Spruce Seal and Vapor Gard (1–5%) Wilt Pruf (5–25%) prevented spore germination and significantly reduced blue stain infection of wood left in the forest for 4 weeks. Each of the four antitranspirants significantly reduced water loss from the cut surfaces of the logs. There was no infection when the water content of the wood was more than 50%.

These observations indicate that successful control of blue stain of wood should be aimed at either depleting the food reserves in the wood or using chemicals that will prevent rapid water loss from cut surfaces of logs.

I am grateful to Dr J. F. Levy of Imperial College of Science and Technology, London who supplied me with *R. atrovirens*, *C. globosum* and *P. fastigiata*.

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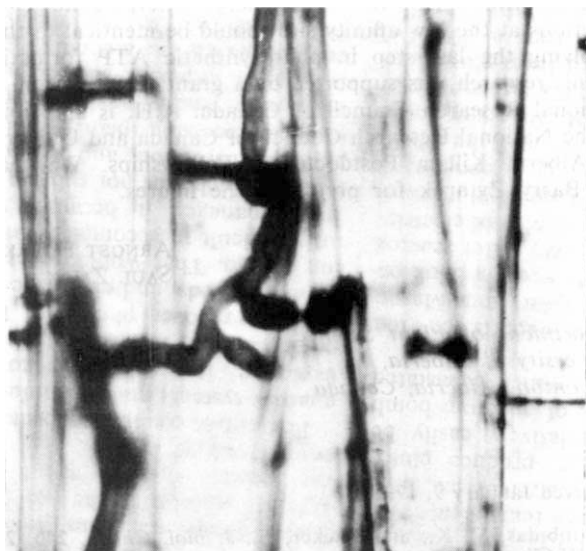


Fig. 1 Brown hyphae in vessels of *Antiaris africana*.  $\times 480$ .

# matters arising

## Spotted centromeres in human chromosomes

SIR,—The demonstration by Eiberg<sup>1</sup> of equal sized Giemsa-stained paired dots in the centromeric regions of human chromosomes, raises the question of whether these dots are equivalent to, or associated with, the paired spheres, or dots, observed in these regions in chromosomes preparations handled in different ways.

Sharply delineated pairs of unstained spheres associated with the centromere are often to be seen in human, mouse and other mammalian chromosome preparations of cells pretreated with colcemid and stained with orcein, or Giemsa, in the conventional way. These spheres stand out more clearly in cells with cytoplasmic staining and they have the appearance of unstained vacuoles (Fig. 1a). Dots, perhaps more closely similar to those described by Eiberg<sup>1</sup>, are also to be seen in optically shadowed, but unstained, human chromosomes<sup>2</sup> that have been exposed to the alkali and saline pretreatments necessary before Giemsa staining in a technique<sup>3</sup> to give C-banded chromosomes, or to a trypsin schedule<sup>4</sup> to give G banding. These dots appear as raised prominences in the centromere regions of the collapsed chromosomes and are similar in size and position in all members of the chromosome complement (Fig. 1b and c). They cannot be simply equated to the densely stained Giemsa C bands, as the size of the centromerically located C bands differs between chromosomes and indeed some C bands are very large (such as those of chromosomes 1, 9 and 16) and are not confined in their location to the centromere regions as, for example, in the Y chromosome. Differences between C bands and a more localised centromeric staining have also been noted by Voiculescu *et al.*<sup>5</sup> in banded preparations of hamster chromosomes. The smaller centromeric C bands in the human complement (Fig. 1d), however, cannot be distinguished from the unstained promontories seen in these chromosomes in shadowed, but unstained, preparations.

The regions in which these stained, or unstained, dots appear are known to contain the paired disk-shaped kinetochores that are located in the centromere region on opposite sides of sister chromatids and which are clearly visible

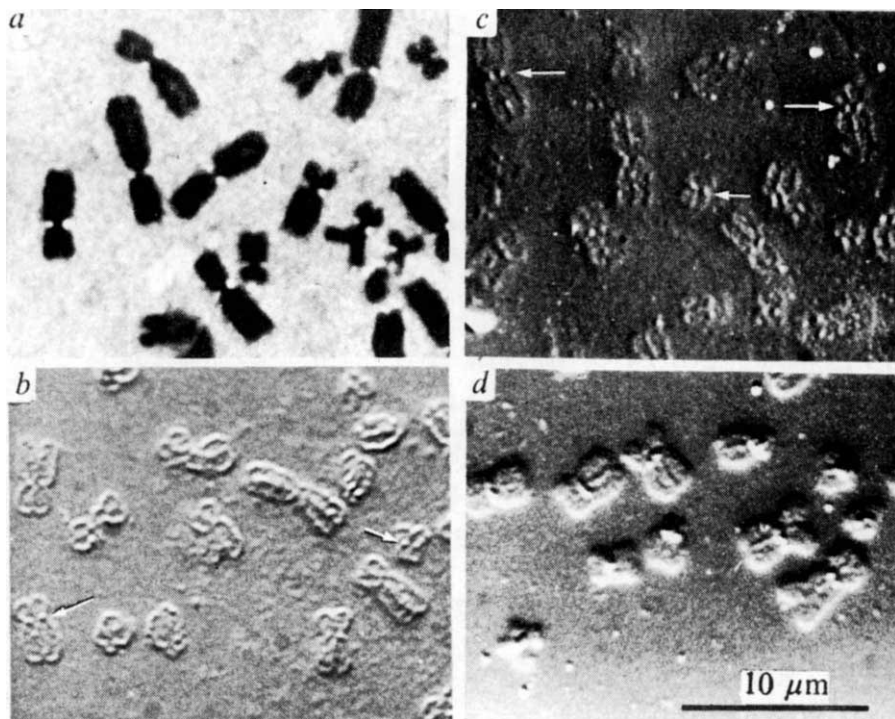


Fig. 1a, Human chromosomes stained with lactic-orcein and showing the pairs of unstained dots associated with the centromere. b, Unstained optically shadowed human chromosomes treated with trypsin as in Seabright's<sup>4</sup> G banding technique. Note the paired dots at the centromeres. c, Unstained and shadowed chromosomes treated with HCl, saline and Ba(OH)<sub>2</sub> as for C banding<sup>3</sup>. Note the paired dots at the centromeres. d, Shadowed chromosomes that have been stained for C banding. Compare the prominence at the centromere with b and c.

using the electron microscope<sup>6</sup>. Such studies have also shown that the paired fibrillar elements of the kinetochore have associated with them a less dense granular matrix which is also fibrillar in nature and which does not completely encircle the centromeric regions of the chromatids<sup>7</sup>. Moreover, although colcemid inhibits the full development of the spindle, the centromerically located spindle tubules are always present so that spindle proteins are concentrated in these regions and attached to the chromosome<sup>8</sup>. It would seem to us therefore that the stained, or unstained, dots which are seen under the light microscope to be specifically located in the centromere regions, may represent the kinetochores and particularly their associated proteins.

It is perhaps worth noting that although in man<sup>9-11</sup> and mouse<sup>12</sup> C band material is confined to the centromere regions in the autosomes, in cultured cell lines these materials are sometimes translocated away from the centromere. Thus, even in these materials, C banding does not provide a reliable method for

detecting centromeres. On the other hand, a simple centromere staining method, such as Eiberg's, would, if consistent, provide a good method for locating centromeres and a useful tool in resolving the centromere states of D/D, D/G and G/G chromosome translocations in man and in screening for dicentric chromosomes in irradiated cells.

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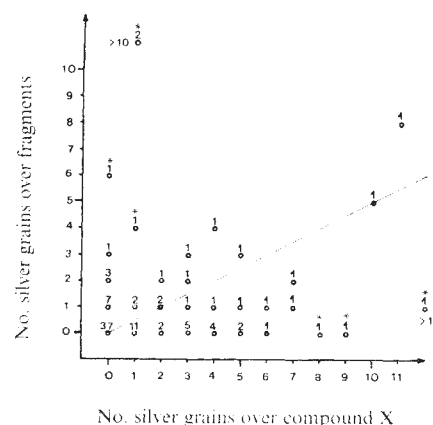
## Random deviations or asynchronous DNA synthesis?

SIR,—In a study concerning the labelling of the X chromosomes of heterozygous Dp(1:f) AM female larvae, Bender, Barr and Ostrowski<sup>1</sup> compared the absolute amount of silver grains distributed over the 1AB region of the paired X chromosomes (compound X) with the absolute amount of labelling over the homologous region of the free duplication (fragment). In all 97 spot-labelled nuclei analysed, the 1AB region of the fragment and the compound X were asynapsed. In 65 of these

On the basis of experiences with autoradiographic studies (ref. 2, and our unpublished results), we wish to mention that there is still another interpretation for these data. During the preparation of our manuscript, similar judgements have been published<sup>3</sup>.

The data published in Fig. 1 of ref. 1 are repeated here, but are tabulated in a different way (Fig. 1 and Table 1). It is noteworthy that only seven (Fig. 1) of 97 nuclei show relatively strong deviations (more than three silver grains) from the axis of synchrony. In three of these cases the fragment synthesises its DNA earlier and in the remaining four later, than the compound X. Eleven nuclei show only one silver grain in compound X and none in the fragment. The remaining 49 labelled nuclei (including the seven exceptions) are distributed with 23 on each side of the axis of synchrony and three nuclei matching the axis of synchrony directly.

In light of the observation that of 97 nuclei with 194 1AB regions, 113 were unlabelled and the remaining 81 were distributed according to Table 1, it seems to be inconsequential, in our opinion to eliminate 44 cases of less than three silver grains as background



**Fig. 1** Deviations of labelling intensity over the two 1AB regions in 97 salivary gland nuclei of Dp(1:f)AM female larvae, according to the data of ref. 1. The position of each circle indicates the absolute number of silver grains over the 1AB regions of the duplication (fragment) observed, as well as over the 1AB region of the two paired X chromosomes (compound X). An axis of synchrony is drawn through those circles which show a 2:1 ratio for the amount of silver grains. Figures on top of each circle indicate the number of cases observed. \*Nuclei with strong deviations of labelling.

**Table 1** Number and distribution of silver grains observed over each of the two 1AB regions in 97 nuclei of Dp(1:f)AM female larvae (changed after Bender, Barr and Ostrowski<sup>1</sup>)

| No. of silver grains observed over the 1AB regions | >10 | 11 | 10 | 9 | 8 | 7 | 6 | 5 | 4 | 3  | 2  | 1  | 0   |
|----------------------------------------------------|-----|----|----|---|---|---|---|---|---|----|----|----|-----|
| Distribution of 1AB regions observed               | 3   | 1  | 1  | 1 | 2 | 2 | 3 | 5 | 8 | 11 | 11 | 33 | 113 |

nuclei a maximum of only two silver grains could be found over each of the two 1AB regions. The authors deemed this amount of labelling as insignificant. Of the remaining 32 nuclei, 22 showed significant labelling over the compound X but an insignificant labelling over the fragment. In contrast, only five nuclei indicated significant labelling over the fragment but insignificant labelling over the compound X. In the remaining five nuclei, significant labelling was found both in the fragment and in compound X as well.

From these results the authors concluded that the fragment does not replicate the DNA in synchrony with the homologous region of the compound X chromosome. This conclusion is based on two assumptions. First, that labelling of less than a tight cluster of three silver grains over a given chromosome region is insignificant and, second, that a synchronous DNA synthesis must be reflected by a constant ratio in the amount of silver grains of the two homologous chromosome sections without any random deviations.

labelling: in particular, because the authors claim that background labelling of the autoradiograms analysed was very low. Recent (unpublished) studies have shown that the restriction in the significance of labelling in respect to a tight cluster of three silver grains must be handled with caution, if slides with a very low background are used.

The authors' assumption, that the silver grains in the two corresponding 1AB regions have to indicate a constant 2:1 ratio according to their DNA amounts (or somewhat less than 2:1, because of the greater self absorption in the compound X), is based on the studies by Plaut and Nash<sup>4</sup>. During (an unpublished) reinvestigation of this problem, however, one of us (K. H.) has shown that the labelling in homologous bands of normally structured asynapsed chromosomes showed only a high degree of correspondence and not the absolute correspondence of Plaut and Nash<sup>4</sup>. Random deviations were to be found with common frequencies (Fig. 1). It must also be mentioned that 6.8–28.8% of 'exceptional patterns' are to be found in all phases of the replication cycle of

normally structured chromosomes in *Drosophila melanogaster*<sup>5–7</sup>. Therefore, the seven nuclei with strong deviations (Fig. 1) may belong to those exceptional deviations. Also, on the basis of the arguments presented, we believe that the replication of the duplicated chromosomal region should better be interpreted as synchronous DNA synthesis.

Yours faithfully,

WOLF-EKKEHARD KALISCH  
KLAUS HÄGELE

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<sup>1</sup> Bender, H. A., Barr, H. J., and Ostrowski, R. S., *Nature new Biol.*, **231**, 217 (1971).

<sup>2</sup> Kalisch, W.-E., and Hägele, K., *Chromosoma*, **44**, 265 (1973).

<sup>3</sup> Rudkin, G. T., in *Results and Problems in Cell Differentiation*, **4**, (Springer Verlag, Berlin, 1973).

- <sup>4</sup> Plaut, W., and Nash, D., in *The role of chromosomes in development*, 113, (Academic, New York, 1964).  
<sup>5</sup> Plaut, W., Nash, D., and Fanning, T., *Molec. Biol.*, **16**, 85 (1966).  
<sup>6</sup> Nash, D., and Bell, J., *Can. J. Genet. Cytol.*, **10**, 82 (1968).  
<sup>7</sup> Lakhota, S. C., and Mukherjee, A. S., *J. Cell Biol.*, **47**, 18 (1970).

- <sup>2</sup> Winterberg, F., *Rev. scient. Instrum.*, **41**, 1756 (1970).  
<sup>3</sup> Hirsch, E. H., *Rev. scient. Instrum.*, **42**, 1371 (1971).  
<sup>4</sup> Winterberg, F., *Rev. scient. Instrum.*, **43**, 814 (1972).

## Magnetic insulation

SIR,—The extravagant claims presented in a recent article by Winterberg<sup>1</sup>, led me to look into the background of the idea of 'magnetic insulation'. The purpose of this letter is to point out that the scheme described in that article is unsound and will not work.

The basic idea in Winterberg's article is the prevention of electrical breakdown between conductors at different potentials using the interposition of a combination of a high vacuum and a high magnetic field parallel to the conductor faces. Electrons which could initiate the breakdown are supposed to be returned by the magnetic field to the electrode from which they were emitted. This, however, is essentially the configuration found in any number of magnetron devices. In forty years of experiments in this field it has been shown time and again that complete cutoff is not achieved for any magnetic field. The reason is that collective effects intervene; space charge instabilities always result in some current passing between the electrodes. Under these conditions simple orbit calculations no longer apply.

Winterberg's ideas were presented earlier in a longer article<sup>2</sup>. In that article he suggested that 'magnetic insulation' might make possible a transformer for 10<sup>9</sup> V. A year later the same objections that I have raised were published in some detail by Hirsch<sup>3</sup>. He concluded "The cutoff anomaly therefore renders 'magnetic insulation' impracticable... Clearly, the only way in which vacuum breakdown due to field emission from a given surface can be avoided is by reduction of the surface electric field." A reply by Winterberg<sup>4</sup> completely failed to answer Hirsch's objections.

Perhaps claimants for 'magnetic insulation' could be given the same requirement that is presented to inventors of perpetual motion machines. They are usually instructed to accompany their claims with a working model.

Yours faithfully,  
JOHN P. BLEWETT

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<sup>1</sup> Winterberg, F., *Nature*, **246**, 299 (1973).

DR WINTERBERG REPLIES: Contrary to Blewett's belief, magnetic insulation has not only been experimentally confirmed<sup>2</sup> since I proposed it several years ago<sup>1</sup>, but is already utilised in the coaxial transmission lines of relativistic electron beam generators (for example, the MJ Aurora machine). The magnetic field needed for the insulation effect in this case is generated by the strong azimuthal self-induced field of the current pulse passing through the transmission line. In the Aurora machine for example, the pulse length is 100 ns and the feasibility of magnetic insulation is therefore confirmed, at least for this time scale. The same should therefore be true for the magnetically insulated high voltage transformer<sup>3</sup>, at least for an a.c. frequency of the order 10 MHz as it is realised in a Tesla transformer.

Blewett argues that experience with magnetron devices speaks against the feasibility of magnetic insulation and that the device described in my article falls into the same category. This, however, is not quite correct, because the magnetron device is an open ended system. It therefore has quite similar end losses and instabilities as an open ended plasma confinement device. This contrasts with a closed toroidal system, which is proposed in my article. In a magnetron the electrons can drift freely in the axial direction, causing breakdown at the ends, by contrast with the proposed closed toroidal system in which this free motion follows a closed loop parallel to the circular torus axis.

Both the magnetically insulated coaxial transmission line and the high voltage transformer are also open ended systems but with an azimuthal field, rather than an axial field as in the magnetron. The azimuthal field leads to a much smaller axial drift motion than in the magnetron device. Here again, the analogy with the z pinch (azimuthal field) and the  $\theta$  pinch (axial field) can be drawn. Therefore, for both the magnetically insulated coaxial transmission line and the high voltage transformer, larger time scales than in the magnetron can be expected. This prevents breakdown. A closed toroidal system, as it is proposed in my article should, however, lead to substantially larger breakdown times, quite analogous to the much longer plasma confinement times for closed than for open ended systems. It is also known, from toroidal plasma confinement devices, that even higher stability can be achieved by strong shearing of the magnetic field lines and average minimum  $B$ , as in the Stellarator or Tokamak. The same principle could, of course, be used

in the proposed machine, a big shear field being produced with the external field coil, with an induced circular current in the central conductor or with a combination of both. In a closed, magnetically insulated system the only important collective instability which then remains is the diocotron instability which is suppressed by making  $\omega_p^2 \ll 2\omega_c^2$  where  $\omega_p$  is the electron plasma frequency and  $\omega_c$  the electron cyclotron frequency. This is one of the conditions for magnetic insulation.

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<sup>1</sup> Winterberg, F., *Phys. Rev.*, **174**, 212 (1968).

<sup>2</sup> Miller, R., Rostocker, N., and Nebenzahl, I., *Bull. Am. phys. Soc.*, **17**, 1007 (1972).

<sup>3</sup> Winterberg, F., *Rev. scient. Instrum.*, **41**, 1756 (1970).

## Bovine thymin

SIR,—Recently, Goldstein<sup>1</sup> reported on the isolation of two polypeptide hormones from bovine thymus. He suggests and extensively uses the terms thymin I and thymin II to designate these hormones.

The choice of these names seems unfortunate because of a possible confusion with thymine (2,4-dihydroxy-5-methyl-pyrimidine), a component of some nucleic acids. In French and German, there would be only one way of writing both thymine and thymin, so that they could not be distinguished from each other. The numbers I and II should also be considered as provisional ones, as the structures of the hormones are still unknown and it could be that II is the precursor of I.

It is to be expected that any new important biochemical compound will ultimately have its name defined on an international basis by a nomenclature commission of such a body as the International Union of Biochemistry (IUB) or the International Union of Pure and Applied Chemistry (IUPAC). As this is likely to take some time, it would be worthwhile if Dr Goldstein could reconsider the question of the names to be used for the new hormones, to make them unambiguous and compatible with existing recommendations.

Yours faithfully,  
M. ROTH

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Hôpital Cantonal,  
Geneva, Switzerland

<sup>1</sup> Goldstein, G., *Nature*, **247**, 11 (1974).

DR GOLDSTEIN REPLIES: The points made by Dr Roth are well taken. The name thymin was originally assigned<sup>1</sup> to designate a hormone of the thymus



by the traditional '-in' suffix (as for insulin and renin). At that time we avoided naming the hormone by its action as we were uncertain whether the neuromuscular effect was physiological; indeed, it now does not seem to be so.

Unfortunately, there is confusion between the polypeptide hormone thymine and the base thymine, which was also named for its original isolation from thymus. Would the name thymopoietin be more acceptable? This would indicate the action of the hormone in inducing stem cell differentiation to, or 'producing', thymocytes (as in erythropoietin and granulopoietin).

I agree that the numbers I and II are provisional and that this will be resolved by structural work, which is in progress.

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<sup>1</sup> Goldstein, G., and Hofmann, W. W.,  
*Clin. exp. Immun.*, **4**, 181-189 (1969).

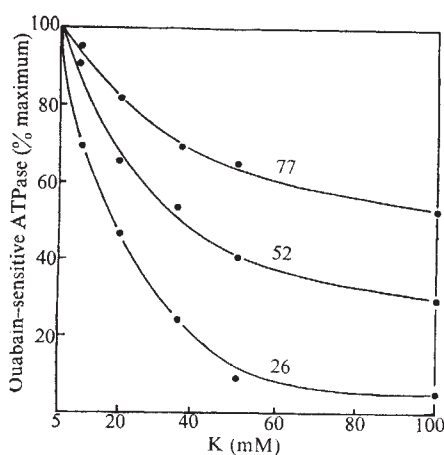
## (Na + K)-ATPase activity in cattle red cells

SIR,—Schatzmann<sup>1</sup> has described a positive correlation between ouabain-sensitive ATPase activity and red cell K levels in cattle, cells with higher K levels giving a greater enzyme activity. These determinations were made with Na=150 mM, K=10 mM. If ouabain-sensitive ATPase is determined as a function of K concentration (at K levels >5 mM to saturate the outside pump site) in fragmented red cell ghosts from individual cattle with different erythrocyte K levels, it can be seen that, as in other ruminant red cells<sup>2</sup>, the enzyme shows a variable degree of K inhibition (Fig. 1). Thus, membranes derived from cells having an original K level of 26 mmol per 1 cells are 50% inhibited at K=20 mM, whereas membranes from cells originally having 77 mmol K per 1 cells are not 50% inhibited even at K=100 mM. From the 26 cows so far investigated a continuous spectrum of K sensitivities has been found, with the degree of K inhibition correlating well with the resting K level of the original red cells. It therefore seems likely that the internal affinity for K of the ouabain-sensitive ATPase in cattle red cells may be the dominant determinant of resting cell K levels, and not the amount of enzyme activity measured at relatively low K concentrations.

Yours faithfully,

J. C. ELLORY

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Babraham, Cambridge CB2 4AT, UK



**Fig. 1** Ouabain-sensitive ATPase activity in cattle red cell ghosts as a function of K concentration. Ghosts prepared and assayed as previously described<sup>3</sup>. Incubation conditions: NaCl 150 mM, MgCl<sub>2</sub> 1.25 mM, Na<sub>2</sub>ATP 1.25 mM, 10 mM Tris pH 7.7 at 37° C, (KCl+choline Cl) 100 mM, ouabain, when present, 0.1 mM. Ghost concentration equivalent to 25% haematocrit, original packed cells. All conditions measured in quadruplicate. Curves shown for cells from three individual cattle with red cell K 77, 56, 26 mmol per 1 cells. Data were normalised as % maximum ouabain-sensitive ATPase activity (at 5 mM K). Actual maximum ouabain-sensitive ATPase activities at 5 mM K; upper curve, (77 mmol K per 1 original cells), ATPase 375 nmol P<sub>i</sub> per mg protein per h; middle curve, (52 mmol K per 1 original cells), ATPase 660 nmol P<sub>i</sub> per mg protein per h; lower curve, (26 mmol K per 1 original cells), ATPase 260 nmol P<sub>i</sub> per mg protein per h.

<sup>1</sup> Schatzmann, H. J., *Nature*, **248**, 58 (1974).

<sup>2</sup> Ellory, J. C., Glynn, I. M., Lew, V. L., and Tucker, E. M., *J. Physiol. Lond.*, **217**, 61P (1971).

<sup>3</sup> Fortes, P. A. G., Ellory, J. C., and Lew, V. L., *Biochim. biophys. Acta.*, **318**, 262 (1973).

DR SCHATZMANN REPLIES: The marked variability of the K affinity of the internal Na site found by Dr Ellory renders untenable my tacit assumption that the ATPase activity measured at 10 mM K and 100 mM Na reflects the number of pump sites per cell. But unless our conditions of Na concentration, pH and ionic strength considerably enhance the effect due to different affinities it does not seem to account completely for the variability of the Na+K-ATPase which we reported. From the regression in our experiments cells with [K]<sub>cells</sub> = 77 mmol l<sup>-1</sup> might be expected to have 4.5 times the ATPase activity of those with 26 mmol l<sup>-1</sup>, whereas the curves shown above only yield a factor of about

1.4 at 10 mM K. Yet I fully agree with the conclusion that the internal K affinity seems more important than the number of pump sites per cell in determining the cellular K concentration.

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## No chromosome 9q+ in Ph<sup>1</sup>-negative CML

SIR,—To demonstrate that in Ph<sup>1</sup>-positive cells the additional band at the end of the long arms of chromosome 9 (9q+) stems from chromosome 22, Rowley<sup>1</sup> suggested the examination of metaphases derived from bone marrow from patients with Ph<sup>1</sup>-negative chronic myelocytic leukaemia (CML). We studied the chromosomes of three such patients, two of whom had symptoms considered to be characteristic for Ph<sup>1</sup>-negative CML<sup>2</sup>, that is, mild leukocytosis, anaemia, severe thrombocytopenia and discrete splenomegaly.

Case 1 died of a cerebral haemorrhage 3 months after first presentation. Case 2 entered blastic phase and died 20 months after CML had been diagnosed; chromosomes were studied during blastic phase. Case 3 has typical CML and is well 3 yr after the diagnosis has been established.

The chromosome banding pattern was obtained by the trypsin-saline-Giemsa method. The karyotype of case 1 was normal, 46,XY. Case 2 had two clones; the major clone (60%) had the karyotype 47,XY, 20q-, +21 and the minor clone (40%) had 46,XY,20q-. In case 3 the marrow cells revealed a missing Y on several occasions; the karyotype was 45,XO while the PHA-stimulated lymphocytes were chromosomally normal.

Chromosome 9q+ could not be detected in any metaphase of the Ph<sup>1</sup>-negative cases. Thus, these findings corroborate Rowley's assumption<sup>1</sup> that the additional material on chromosome 9 comes from chromosome 22. Furthermore, an abnormal banding pattern which would characterise Ph<sup>1</sup>-negative CML could not be seen. This may be related to the finding that patients with Ph<sup>1</sup>-negative CML represent a rather heterogeneous group<sup>3</sup>.

Yours faithfully,

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<sup>1</sup> Rowley, J. D., *Nature*, **243**, 290-293 (1973).

<sup>2</sup> Ezdinli, E. Z., Sokal, J. E., Crosswhite, L. H., and Sandberg, A. A., *Ann. int. Med.*, **72**, 175-182 (1970).

# book reviews

## Builders of the Universe

*The Astronomical Revolution: Copernicus–Kepler–Borelli.* By Alexandre Koyré. Translated by R. E. W. Maddison. Pp. 531. (Hermann: Paris; Methuen: London; Cornell University: Ithaca, July 1973.) £6.50.

*The Cosmology of Giordano Bruno.* By P. H. Michel. Translated by R. E. W. Maddison. Pp. 306. (Hermann: Paris; Methuen: London; Cornell University: Ithaca, July 1973.) £4.50.

THE writings of the late Alexandre Koyré never fail to reveal the intimacy of the union between the history and the philosophy of science. His interpretation of *The Astronomical Revolution*, first published in French in 1961 and now in an English translation is, to quote the author's own expression "the history of the evolution and transformation of the key concepts" (page 9) in astronomy as opposed to a history of astronomy. This distinction arises from the fact that Koyré took no account of advances in observational methods and techniques, since he was of the opinion that these had virtually no influence upon the metaphysical revolution which he is consciously seeking to epitomise through Copernicus, Kepler, and Giovanni Alfonso Borelli. He has, as it were, defined practical astronomy out of existence as being irrelevant to his purpose of exposing both "hidden and acknowledged motives and incentives" (page 18) in the literary legacies of these three men.

The fact that he himself regarded this dichotomy between theory and observation as "rather strange" (page 9) indicates that he might have been prepared to concede the importance of observational astronomy if he had not been bound by his own ontological terms of reference. He cites Rheticus's view that it was Copernicus's success in explaining the lunar theory without equants which encouraged him to carry his explanation further, without stressing that the latter's initial dissatisfaction with Ptolemy's lunar theory was founded upon the observation that the Moon's parallax and angular diameter "differ little, or not at all, from those that are observed at conjunction and opposition" (page 40). Though no one would deny that Copernicus's own observations seem to have been few and their accuracy no better than those of Ptolemy, there are some who are

not prepared to accept the inference that observational astronomy had little significance upon the former's belief in the Earth's axial rotation (see J. R. Ravetz, *Scientific American*, **215** 88-98, 1966) Since Koyré placed his entire emphasis upon the heliocentric hypothesis and neglected the question of the Earth's diurnal motion he cannot be challenged on this score, but one might well query the justification of according such an unequal status to these historically inseparable axioms of Copernican astronomy.

The author's interpretation of Kepler's achievements occupy roughly two thirds of this book. Kepler's discovery that the planetary orbits are elliptical with the Sun at one focus—his first law—is attributed to the fact that he was trying to explain discrepancies between theory and observation within the framework of a celestial physics conceived long before he had access to Tycho Brahe's data. On this ground the latter are regarded as having been of little importance to Kepler's rejection of Ptolemy's time-honoured kinematical model of circles and spheres. It is nevertheless true that Kepler derived all three of his laws empirically after years of trial and error in trying to fit Brahe's planetary observations to his physically erroneous model of the solar system. Had the observations of Mars, in particular, been intrinsically less reliable, more dispersed spatially, or more restricted temporally, Kepler—despite his unquestionable genius—would now be featuring much less boldly in the history of science.

The significance attached by Koyré to Borelli is due principally to the latter's recognition that circular motions produce centrifugal forces. Here the thought is considered to be more important than the deed, since Borelli's speculations in his *Theoriae Mediceorum Planetarum* (Florentiae, 1666) were not sufficiently clear to lead to any predictable results. He did not analyse the phenomenon of curvilinear motion into its constituent components and gave no explanation of the origin of the centrifugal tendency, merely seeking to establish the necessity of a constant central force by an appeal to experiment. Koyré sees in him a true disciple of Galileo, who adopts the same methodological approach of using theory to guide experiment "which confirms, or invalidates, the theory, and provides firm data relating to the matter under investigation" (page 469).

Stillman Drake has recently cast doubts upon whether this image of Galileo which Koyré himself, in *Études galiléennes*, has done much to propagate, comprehends a true reflection of his epistemology (see *Scientific American*, May, 84-92; 1973) but whether this will affect future assessments of Borelli's work is difficult to say. The latter's deductive approach is here attributed to "the sterility of observation, pure and simple" (page 471), and the telescopic observations which he had been making for years on Jupiter's satellites are supposed merely to have provided an excuse for publishing his theory of their motions. But one cannot escape the fact that this theory was developed from the ellipticity of planetary orbits—a 'fact' derived observationally by Kepler and accepted by Borelli on observational grounds (as an extract from his writings quoted on page 474 clearly reveals). Thus here again it ought to be recognised that Koyré's philosophical distinction, though heuristically convenient, is historically inadequate.

In another of his writings, Koyré once claimed that the greatest achievement of the scientific revolution was the destruction of the cosmos which it had been the greatest achievement of the Greeks to create. The determinative factor in this dramatic ontological transformation was the collapse of the finite bounded Aristotelian Universe and the consequent rejection of a central point. This notion originated not from Copernican astronomy but from an admixture of neoplatonic philosophy and commentaries such as that of Nicolas of Cusa upon the Aristotelian vision of the heavens. *The Cosmology of Giordano Bruno* is concerned with this metaphysical revolution which precedes yet transcends the astronomical revolution of Copernicus, Kepler and Borelli. Even the Sun loses its privileged position in an infinite Universe, but the Copernican (and Pythagorean) postulate of the Earth's diurnal rotation can be seen as a logical corollary to the observed fact that all the stars seem to be carried round the heavens at the same uniform speed in a westerly direction. Thus a belief in Bruno's cosmology, with its emphasis on the infinity of the Universe might have acted as a stimulus to the acceptance of the new astronomy, and *vice versa*. Here, however, the late Paul-Henri Michel is not interested in exploring possible connections between the seventeenth





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## Journal of Neurocytology

EDITORS: E. G. GRAY and  
A. R. LIEBERMAN, both in  
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University College London.

The policy of the *Journal* is to publish, with minimum editorial and production delay, high-quality research papers, analytical reviews, and occasional letters. In order to achieve this aim more completely the journal will be published as a bi-monthly rather than a quarterly journal from the beginning of 1974. Papers deal with fine structural studies and associated cytochemical, biochemical, physiological and pharmacological studies of neurons, receptors, synapses, neuro-effector junctions, glia and other elements of the peripheral and central nervous system. Studies of both vertebrate and invertebrate nervous systems under normal, experimental, and pathological conditions are all suitable for the *Journal*.

Important features are the editorial emphasis on adequate illustration of papers and the publisher's concern for high-quality reproduction of plates.

Annual Subscription 1974 (6 issues): U.S.A., Canada and Mexico \$49.50 U.K. and all other territories £18.75. Full details and a specimen copy are available on request.

## Topology and Normed Spaces

G. J. O. JAMESON,

*Chapman & Hall Mathematics Series*

Hardback: June 1974: 424 pages: £5.80 Limp cover: £3.80 Based on courses given at the Universities of Warwick and Innsbruck, this book provides a general introduction to topology and then uses topology to introduce normed spaces. Normed spaces provide the most natural examples of topological spaces and also set the scene for many of the best applications of the theorems of general topology. This approach should be invaluable for students who have done some elementary analysis and linear algebra.

Further details of mathematical books and a list of stockists is available from the publishers on request.

century reaction against Bruno's heresies and the slow initial reception of Copernican astronomy, but rather with examining the internal consistency of the cosmological ideas in Bruno's writings; it being the central thesis of his book that the latter embody a systematic philosophy of nature. The work is consequently likely to have a greater appeal to philosophers of science than to historians of science specialising in Copernican studies.

Though drawing upon statements from all Bruno's extant cosmological and moral writings—these date from 1582 to 1592—the author is concerned principally with the contents of the three vernacular dialogues *La cena de le ceneri*, *De la causa, principis et uno*, *De l'infinito immenso et mondi* of 1584, and the trilogy of Latin poems *De minimo*, *De monade*, and *De immenso* composed seven years later. These, in his opinion, "have in common a scholarly, careful and almost meticulous method of utterance" (page 50). Yet his attempts to justify his belief in the compatibility of Bruno's statements require all his powers of exigesis and are at times less than convincing. For example, he dismisses an inconsistency between Bruno's views in *De la causa* and the moral treatise *Eroica furori* regarding the possibility of attaining supreme metaphysical knowledge, as resulting from "two complementary aspects of a single consistent thought, rather than two different modes" (page 58). He confesses that Bruno's ideas concerning the indivisibility of the soul are "scattered, and sometimes contradictory" (page 116); admits that Bruno's answer to the question of continuity in the material Universe "was not vigorously asserted" (page 132); and tells us that "we must resign ourselves" to the fact that Bruno's conception of an aether "retains its ambiguous and mysterious character" (page 140). Moreover, he remarks upon Bruno's "unfitness for certain kinds of mathematical reasoning" (page 145); and so on. It would seem much less difficult to defend the opposing thesis that Bruno was not quite up to the task of producing a consistent poetic, polemic, and aphoristic description of the Universe; and that his philosophical utterances on its infinity and homogeneity, the plurality of worlds, and animism in nature, were little more than a regurgitation of ill-digested ideas from Albertus Magnus's and St Thomas Aquinas's discussions of the doctrines of David of Dinant, and from works by Nicolas of Cusa and Marsilio Ficino.

Dr Maddison's translations of both Koyré's and Michel's books are excellent, and misprints are few. In the former, the year of Tiedemann Giese's

letter to the magistrates of Nuremberg complaining of Osiander's breach of faith to Copernicus is wrongly printed on page 37 as 1523 (instead of 1543). In the latter (page 36), the twentieth century French scholar Etienne Gilson is said to have been writing in 1628, and a rather odd mistake on the book jacket is the statement that Bruno was burnt as a heretic in Rome in 1589 (rather than 1600). But these are but minor errors. English-speaking historians and philosophers of science will undoubtedly be pleased to have these well documented studies of sixteenth and seventeenth century thought accessible in their native language, at relatively modest prices.

ERIC G. FORBES

## Rockefeller Research

*The Arthropod-Borne Viruses of Vertebrates: An Account of the Rockefeller Foundation Virus Program, 1951-70.* By M. Theiler and W. G. Downs. Pp. xxviii + 578. (Yale University: New Haven and London, June 1973.) \$25; £10.50.

THE agent causing yellow fever was shown to be a virus only in 1927. By 1950 about 20 other arthropod viruses, or arboviruses, had been discovered, mostly as a result of work primarily on yellow fever, but now more than 300 such viruses are known. This great increase in the numbers of known arboviruses is largely due to the Division of Medicine of the Rockefeller Foundation. Workers from here first studied yellow fever in 1914 and played a major part in the isolation of the virus, in elucidating the epidemiology of the disease, and in devising control measures, recognised by the award of a Nobel Prize in 1951 to the late Dr Theiler for work on the development of an effective vaccine.

Because of a lack of knowledge about the significance of arboviruses other than yellow fever in causing morbidity and mortality in man or his domestic animals, the foundation decided in 1950 to start a major research programme to investigate them, particularly in tropical countries where they are prevalent. In addition to the existing yellow fever laboratories supported by it in Entebbe, Lagos and Rio de Janeiro, the foundation, over several years, established new field laboratories in Poona, Cairo, Johannesburg, Ibadan, Port of Spain, Belem and Cali. Although staff members were assigned to these laboratories, the subprofessional staff were recruited almost entirely from local people, and an integral part of the programme was the training of workers from the coun-

tries themselves so that they could eventually take over the laboratories. As well as financial support, travel grants and fellowships were given to laboratories working on arboviruses in many other countries.

The Rockefeller Foundation Virus Laboratory, New York, which moved to Yale in 1964 as the Yale Arbovirus Research Unit, acted as a coordinating laboratory, as a centre for training and for the evolution of new techniques, and as a reference laboratory for identification and classification of the spate of new arboviruses which were isolated. This intensive research continued for a period of some 20 years but, with a change of emphasis in the activities of the foundation, has recently been curtailed, some would think prematurely.

The purpose of this book is chiefly to give an account of the activities of the central and, to a lesser extent, of the field laboratories. Part 1, consisting of four chapters, deals with the development, application, and interpretation of the classical techniques of neutralisation, haemagglutination-inhibition, and complement fixation tests as applied to the diagnosis of arbovirus infection in man and animals and the identification of isolates. Previously unpublished material is used to illustrate some points but scant reference is made here, or elsewhere, to the development of new techniques such as the use of mammalian or insect cell cultures for arboviruses.

In the second and largest part, comprising ten chapters, the principles of the method of classification of arboviruses into antigenic groups, evolved at the Rockefeller Foundation Laboratories, is explained. This is followed by a systematic description of most of the arboviruses known at the time of writing, from Group A to the ungrouped viruses. Details are given of the isolation, of the classification and of the epidemiology of the viruses in man and animals, as far as this is known, and of their importance as pathogens. Many are still in search of a human disease with which they can be identified. In the third part, of seven chapters, there are general discussions about the world distribution of arboviruses, and of their vectors and vertebrate hosts, which give useful summaries. These are followed by brief discussions of their significance for man and clinical manifestations. Finally, the specific problems of dengue and yellow fever in the Caribbean are described.

As a monument to the notable work of the Rockefeller Foundation in the field of arboviruses this book fulfils its primary objective. As a reference book for the serious worker it is less satisfactory. Much is omitted about fundamental work, some of the epidemiology is dealt with perfunctorily, and there is little reference to recent manifestations

such as the dengue shock syndrome. The most useful sections of the book are the second part and some of the third which, with the extensive bibliography, are ready sources of knowledge.

C. C. DRAPER

## Pituitary

*The Pituitary Gland: A Comparative Account.* By R. L. Holmes and J. N. Ball. Pp. x+397. (Biological Structure and Function.) (Cambridge University London, February 1974.) £10; \$28.50.

THE first chapter of this book deals briefly, but adequately, with a general appraisal of the embryology, morphology and physiology of the pituitary gland. The next few chapters deal with morphology and cytology of the various parts of the gland in mammals beginning with the pars distalis. Perhaps the most confusing aspect of pars distalis cytology lies in the nomenclature used to describe different cell types. Although a functional name is to be preferred, for example thyrotroph for TSH cells, in many cases this is not possible, and the use of other systems of nomenclature, though no fault of the authors, makes this a difficult chapter to read.

A major fault in this chapter, which also deals with staining techniques, and the ultrastructure of the pars distalis, lies in the omission of any reference to the use of the peroxidase-labelled antibody method for identifying cell types. This technique has been widely used in both light and electron microscope studies in the last few years.

The beauty of the opening colour plate whets the appetite for the subsequent micrographs; the identification of cell types in electron micrographs is certainly adequately described, but nearly all of the electron micrographs are of poor quality.

Chapter 4 deals with the pars tuberalis and pars intermedia, serving to point out the lack of knowledge of these two subdivisions of the pituitary. A general outline of the neurohypophysis, which includes the median eminence, follows in chapter 5, with chapter 6 covering the basis for a neural control of the pars distalis. There is a certain amount of repetition of chapter 1 here, but the objective approach of the authors makes this chapter very useful for anyone looking for a brief appraisal of the historical and comparative evidence, for and against, a neurovascular control of pars distalis function.

The next eight chapters are concerned with a comparative account of pituitary structure and function, with a greater emphasis on structure, and

it is in these chapters that the book becomes an invaluable information source for both research workers and teachers. The importance of the portal vessels is not diminished, but to be able quickly to refer to those members of the animal kingdom which have an anterior pituitary gland, but no portal vessels, demonstrates that our dogmas sometimes need deflating.

In each of these eight chapters, information is divided under subheadings; for example in the chapter on teleosts a general paragraph is followed by sections covering the overall structure in several teleost species. This is followed by a description of the histophysiology of the pars distalis, with very useful sections devoted to different cell types from both a morphological and a physiological viewpoint. Hypothalamic control of the teleost pars distalis is then considered from the physiological viewpoint, with several subsequent pages devoted to the pars intermedia. A consideration of neural lobe and hypothalamus follows, and anatomical aspects of hypothalamic control of the anterior pituitary end the chapter. I feel that if these subdivisions had been tabulated, either in the list of contents, or at the beginning of each chapter, the use of this book as a reference source would have increased further.

But any book which goes into such detail in describing the pituitary gland in vertebrate groups from fish to man will be a useful addition to libraries.

H. M. CHARLTON

## Not only frog and rabbit

*Comparative Vertebrate Morphology.* By Douglas and Molly Webster. Pp. xiv+517. (Academic: New York and London, January 1974.) \$32. £15.35.

THIS is an interesting new book on vertebrate morphology, set in clear, readable type and well illustrated. The adjective "comparative" in the title is fully justified, each organ system being illustrated for an example of each extant class or order, as appropriate, and the text similarly trying to cover each extant group. The book certainly does not follow the usual 'dogfish-frog-rabbit' format. Moreover, the text is heavily slanted towards a functional morphological approach, which will undoubtedly make more interesting reading for students than several of the rival texts. The book concentrates on extant vertebrates, though the phylogenetic relationships expressed by current palaeontological opinion are fully appreciated, and used frequently in diagrams. There are a



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few minor errors, for example the ball-and-socket joint is a characteristic of many placoderms, not just *Dinichthys* (page 22), and the description of the mole's digging musculature (page 146) is decidedly misleading. But errors seem to be reassuringly few in number, and the book can at least be commended as an informative text.

The preface says that the book has been written for students attending a one semester course on vertebrates, and before one can recommend it as such, it must be considered in relation to its rivals, especially Romer's *Vertebrate Body* which I recommend to my students. In this comparison, the Webster's book scores heavily on its functional approach—Romer is by contrast rather dry reading. Romer's book is, not surprisingly, better grounded in palaeontology, and gives a better account of the skeletal systems. The Websters on the other hand, give a better account of the nervous system. In value, however Romer's book is so superior that the comparison is hardly worth making—it contains about 35% more figures, 16% more pages, a much better bibliography, yet costs only 30% of the Webster's book. It is difficult to know quite why this new text should be so expensive, but the 45 mm margins to the text are wasteful and the insert of six unhelpful colour plates seems an extravagance. Romer's use of colour on ordinary text figures is far more satisfactory.

In summary, then, this text is sufficiently different from its rivals to warrant a place in university libraries. On simple economic arguments, it cannot possibly be commended to students.

D. W. YALDEN

## Snake bite

*Toxicology and Pharmacology of Venoms from Poisonous Snakes.* by John H. Brown. Pp. xiv+184. (Thomas: Springfield, September 1973.) \$13.75 cloth; \$9.95 paper.

I FIND little merit in this book. For one thing the production is poor. The reproduction of most of the 84 colour plates is bad, of some very bad and of two upside down. It is claimed to be encyclopaedic but the widespread and dangerous viper, *Echis carinatus*, receives scant mention and none in the chapter on symptomatology and treatment. Snakebite in England is mentioned but not in the subcontinent of India!

I cannot make out to whom the book is addressed. The chapter on pharmacology is in technical language and con-

tains information likely to interest only research workers. The factors which make the distinctions between those known to be dangerous, those only potentially dangerous and harmless snakes are only vaguely indicated. There is a full list of poisonous snakes around the world but not a hint on how to go about identifying a specimen. Those who do not already know snakes will learn little.

The best parts deal with yields and preparation of venom and assessment of toxicity. These seem, however, unlikely to justify the purchase price to many readers.

GARTH UNDERWOOD

## Above the atmosphere

*Radio Wave Propagation.* By Armel Picquenard. Pp. vi+343. (Macmillan: London and Basingstoke, April 1974.) £10.

THIS year is the fiftieth anniversary of the experimental proof of the existence of the ionosphere, so that there is a special interest in the subject of Professor Picquenard's new book. It is refreshing to be able to welcome such a book from a Brazilian author, when there have been so many books on related subjects from more northerly parts of the American continent.

The whole range of subjects in radio propagation is reviewed. The early chapters are concerned with the troposphere, including both stratified media and scatter propagation, and with the Earth's surface, including both smooth and irregular terrain and diffraction by obstacles. The chapter on the ionosphere deals with its formation and temporal variation and discusses propagation mainly for an isotropic plasma.

The object is to show how to assess a given radio communication link. More than one third of the book is devoted to the last chapter entitled "Practical Calculation of Radio Links", but much of this space is occupied by charts and diagrams, for example a series of 24 maps of the world showing how the intensity level of atmospherics varies with frequency and with time of day, through the year.

This book is for the practical communications engineer who wants to know the answers and is prepared to take the theory on trust. It is not for the more serious scientist who wants to understand the theory and apply it to new situations. There are errors in some of the formulae although many of them are only minor. On the whole it is a useful book.

K. G. BUDDEN

# Announcements

## Awards

**H. H. Lamb** has been given the **Murchison Award** of the Royal Geographical Society for his work in the field of climatic change.

**Graham Highman** has been awarded the **De Morgan Medal** and **Paul M. Cohen** the **Senior Berwick Prize** of the **London Mathematical Society**.

## Errata

In the article "Effects of bicuculline on functions of inhibition in visual cortex" by D. Rose and C. Blakemore (*Nature*, **249**, 375; 1974) the following corrections should be made: the last sentence of para 1 on page 376 should read 'The erratic mechanical stability of the preparation for topical application used in some experiments, has meant that few cells have been fully analysed so far.'; line 12, para. 6 should read '... best orientation. This suggests the presence ...'; and in Table 1, in the column "cortical layer" the first entry should be VI, the third V and the fourth IV.

In the article "Do D-glucosamine and D-galactosamine form a part of the specific receptor sites for the competence substance on the cell wall of *Pneumococcus*?" by M. Kohoutova and J. Kocourek (*Nature*, **247**, 277; 1974) the first sentence of the paragraph under Fig. 4 should read 'The presence of a 2-N-acetamido-4-amino-2,4,6-trideoxyhexose was also reported in the C substance<sup>13</sup>, and not as printed.

In the article "Behavioural and physiological bases of drinking inhibition in water deprived rats" by E. B. Blass and W. G. Hall (*Nature*, **249**, 485; 1974) the first word of the fourth column of Table 1 should be drink and not pre-load.

## International meetings

August 25-30, **9th Meeting of the Federation of European Biochemical Societies** (the Secretariat of the 9th FEBS Meeting, H-1502 Budapest, P.O. Box 7, Hungary).

August 27-September 3, **International Symposium on Discharge of Sewage from Sea Outfalls** (Water Pollution Research Laboratory, Elder Way, Stevenage, Herts SG1 1TH, UK).

September 1-6, **International Symposium on Bread** (James Hadley, Executive Director, The Rank Prize Funds, 152 Grosvenor Road, London SW1V 3JL).

September 1-6, **15th Congress of the International Society of Haematology** (Fifteenth Congress, International Society of Haematology, P.O. Box 12060, Jerusalem, Israel).

September 1-7, **1st Intersectional Congress of the International Association of Microbiological Societies** (Dr Daizo Ushiba, Secretary General, First Intersectional Congress of IAMS, c/o Science Council of Japan, 7-22-34, Roppongi, Minato-ku, Tokyo 106, Japan).

September 1-14, **UNESCO-UNDP-ICRO Training Course on The Cell Nucleus** (Professor F. B. Straub, Biological Research Center, Hungarian Academy of Science, H-6701 Szeged, P.O.B. 521, Hungary).

September 2-6, **3rd International Congress on Photosynthesis** (Photosynthesis Secretariat, Department of Biochemistry, Weizmann Institute of Science, Rehovot, Israel).

September 2-6, **Residential School Binding Processes Involving Biopolymers** (Miss J. M. Usher, The Chemical Society, Burlington House, Piccadilly, London W1V 0BN).

September 2-6, **6th Conference on Molecular Spectroscopy** (Mr C. H. Maynard, The Institute of Petroleum, 61 New Cavendish Street, London W1M 8AR).

September 2-7, **British Association for the Advancement of Science Stirling Annual Meeting** (British Association, 23 Saville Row, London W1X 2AA).

September 2-7, **4th International Congress on Hormonal Steroids** (Congress Secretariat, Dr J. L. Mateos, Apartado postal 73-132, México, D.F.).

September 2-7, **12th International Congress for Fat Research** (Società Italiana per lo Studio delle Sostanze Grasse, Twelfth ISF Congress, c/o Stazione Sperimentale Oli e Grassi, Via G. Colombo 79, 20133 Milano, Italy).

September 3-6, **5th Canadian Medical and Biological Engineering Conference** (Dr Chas. A. Laszlo, Technical Program Chairman, Bio-Medical Engineering Unit, McIntyre Medical Sciences Center, McGill University, 3655 Drummond St, Montreal, Quebec, H3G 1Y6, Canada).

September 4-6, **Conference on Elementary Particle Physics at Ultra High Energies** (Dr V. A. Bull, Department of Physics, Westfield College (University of London), Kidderpore Avenue, London NW3 7ST).

September 6-9, **Course on the Biological Basis of Ageing** (Director of Special Courses, Department of Adult Education and Extramural Studies, University of Leeds, Leeds LS2 9JT, UK).

September 7-10, **28th Annual Meeting of The Society of General Physiologists** (The Society of General Physiologists, Box 257, Woods Hole, Massachusetts 02543).

September 8-10, **International Symposium on Foams** (Dr D. Seaman, Imperial Chemical Industries Ltd, Jealott's Hill Research Station, Bracknell, Berkshire RG12 6EY, UK).

September 8-13, **National Meeting of The American Chemical Society** (American Chemical Society News Service, 1155 16th St., N.W. Washington D.C. 20036).

September 8-14, **1st International Congress of Ecology** (Secretariat 1st International Congress of Ecology, P.O. Box 9000, The Hague, The Netherlands).

September 8-14, **International Solvent Extraction Conference, 1974** (Dr A. Naylor, British Nuclear Fuels Limited, Windscale and Calder Works, Seascale, Cumberland, CA20 1PG, UK).

September 9-11, **Neurophysin Proteins: Carriers of Peptide Hormones** (New York Academy of Sciences, 2 East 63rd Street, New York 10021).

September 9-11, **4th Meeting of the European Connective Tissue Clubs** (Dr A. J. Bailey, Agricultural Research Council, Meat Research Institute, Langford, Bristol BS18 7DY, UK).

September 9-11, **International Symposium on Radiation Protection in Mining and Milling of Uranium and Thorium** (Symposium Joint Secretariat, c/o Occupational Safety and Health Branch, International Labour Office, CH-1211 Geneva 22, Switzerland).

September 9-11, **17th International Conference on the Biochemistry of Lipids** (Dr Giovanni Galli, Institute of Pharmacology and Pharmacognosy, University of Milan, Via A. Del Sarto 21, 20129 Milano, Italy).



September 9-12, **3rd International Conference Gas Discharges** (Conference Department, Institution of Electrical Engineers, Savoy Place, London WC2R 0BL).

September 9-13, **7th International Conference on Water Pollution Research** (SOCFI, 7 rue Michel Ange, 75018 Paris).

September 9-13, **4th International Symposium on Medicinal Chemistry** (Secretariat 4th International Symposium on Medicinal Chemistry, c/o Merck Sharp and Dohme B.V., Waarderberg 39, P.O. Box 581, Haarlem, The Netherlands).

September 9-14, **18th Ampere Congress on Magnetic Resonance and Related Phenomena** (Professor E. R. Andrew, Department of Physics, The University, University Park, Nottingham NG7 2RD, UK).

September 10-14, **Biostereometrics '74 Symposium** (Dr R. E. Herron, Biostereometrics Laboratory, Texas Institute for Rehabilitation and Research, Baylor College of Medicine, 1333 Moursund Ave., Houston, Texas 77025).

September 10-14, **3rd International Conference on Ferroelectricity** (Dr H. Montgomery, Physics Department, The University, King's Buildings, Mayfield Road, Edinburgh EH9 3JZ, UK).

September 11-14, **2nd International Congress of Collegium Internationale Activitas Nervosae Superioris** (2nd International Congress of CIANS, Czechoslovak Medical Society, J. E. Purkyne, Sokolska 31, 120 26 Praha 2, Czechoslovakia).

September 13-14, **Conduction Electron Scattering in Metals** (The Meetings Officer, The Institute of Physics, 47 Belgrave Square, London SW1 8QX).

September 15-21, **3rd International Congress on Muscle Diseases** (Professor J. N. Walton, Charman of the Organising Committee of the Third International Congress on Muscle Diseases, Regional Neurological Centre, Newcastle General Hospital, Newcastle, UK).

September 15-21, **11th International Congress on High Speed Photography** (11th International Congress on High Speed Photography, c/o Central Unit for Scientific Photography, Royal Aircraft Establishment, Farnborough, Hants, UK).

September 16-18, **International Symposium Theory and Practice of Emulsion Technology** (Dr D. Seaman, Imperial Chemical Industries Ltd, Jealott's Hill Research Station, Bracknell, Berkshire, RG12 6EY, UK).

September 16-20, **5th European Symposium on Fluorine Chemistry** (Dr John G. Gibson, The Chemical Society, Burlington House, London W1V 0BN).

September 16-21, **6th International Conference on Magnetic Resonance in Biological Systems** (Professor K. Wüthrich, Institute of Molecular Biology and Biophysics, ETH, 8049, Zürich, Switzerland).

September 16-21, **6th L. H. Gray Conference on the Initial Shapes of Cell Survival Curves: Clinical and Theoretical Implications** (Dr Tikvah Alper, Medical Research Council, Experimental Radiopathology Unit, Hammersmith Hospital, London W12 0HS).

September 17-19, **3rd European Conference on Hard Magnetic Materials** (Professor K. Hoselitz, Mullard Research Laboratories, Cross Oaks Lane, Redhill, Surrey RH1 5HA, UK).

September 17-19, **Stress Vibration and Noise Analysis in Vehicles** (T. H. Richards, Department of Mechanical Engineering, University of Aston, Gosta Green, Birmingham B4 7ET, UK).

September 17-20, **International Symposium on Practical Applications of Ergonomics in Industry, Agriculture and Forestry** (Organising Committee for the International Symposium on Ergonomics, Ministry of Labour, Str. Scaune No. 1-3, Bucharest, Rumania).

September 18-19, **Electrical Properties of Polymers** (The Meetings Officer, The Institute of Physics, 47 Belgrave Square, London SW1X 8QX).

September 18-19, **Seminar on Electrostatics: Fundamentals, Application and Hazards** (The Meetings Officer, The Institute of Physics, 47 Belgrave Square, London SW1X 8QX).

September 18-20, **Interim Colloquium on Field Simulation** (Dr Yakup Paker, Polytechnic of Central London, 115 New Cavendish Street, London W1M 8JS).

September 18-27, **General Assembly of International Council of Scientific Unions** (Associate Secretary General of IUPAP, c/o Institute of Theoretical Physics, Fack, S-402 20, Göteborg 5, Sweden).

## Reports and Publications

### Great Britain

- The Ciba Foundation for the Promotion of International Cooperation in Medical and Chemical Research. Annual Report for 1973. Pp. 58. (London: The Ciba Foundation, 1974.) [15]
- International Tin Research Council. Annual Report for 1973. Pp. 48. (Greenford, Middx.: Tin Research Institute, 1974.) [15]
- Bulletin of the British Museum (Natural History). Entomology. Vol. 30, No. 3: A Catalogue of the Family-Group and Genus-Group Names of the Coleophoridae (Lepidoptera). By K. Sattler and W. G. Tremewan. Pp. 183-214. (London: British Museum (Natural History), 1974.) £1.50. [25]
- Philosophical Transactions of the Royal Society of London. A: Mathematical and Physical Sciences. Vol. 276, No. 1257: The Place of Astronomy in the Ancient World—a Discussion. Edited by F. R. Hodson. Pp. 1-276. Vol. 276, No. 1258: Derivation and Testing of a Molecular Orbital Description of Ligand Field Spectra. By B. D. Bird, E. A. Cooke, P. Day and A. F. Orchard. Pp. 277-339. (London: The Royal Society, 1974.) [25]
- The Year Book of the Royal Society of London 1974. (No. 78). Pp. 466. (London: The Royal Society, 1974.) £2.10. [35]
- Proceedings of the Royal Irish Academy. Vol. 74, Section A. No. 6: Generalized Shape Theory. By T. Porter. Pp. 33-48. 38p. No. 7: The Conformal Properties of a Poincaré Invariant Field Theory. Pp. 49-60. 26p. No. 8: On the Expansion of Closed Universes. By P. Yodzis. Pp. 61-66. 18p. Vol. 74, Section B. No. 6: The Donegal Granite—a Gravity Analysis. By D. G. C. Young. Pp. 63-74. 21p. No. 7: The Lower Palaeozoic Stratigraphy of the Northern Part of the Leinster Massif. By P. M. Bruck, T. L. Potter and C. Downie. Pp. 75-84 + plate 2. 27p. No. 8: Littoral and Benthic Investigations on the West Coast of Ireland—III. (Section A: Faunistic and Ecological Studies). The Bivalves of Galway Bay and Kilkerrin Bay. By B. F. Keegan. Pp. 85-123. 50p. (Dublin: Royal Irish Academy, 1974.) [75]
- Strategic Survey 1973. Pp. 104. (London: The International Institute for Strategic Studies, 1974.) [85]
- Central Electricity Generating Board. Annual Report 1972-73. Vol. 2: Statistical Digest and Detailed Accounts. Pp. 43. (London: CEGB, 1974.) £1 net. [95]

### Other Countries

- Canada: Department of Energy, Mines and Resources. Coal in Canada. Pp. 36. (Ottawa: Information Canada, 1974.) [295]
- Smithsonian Contributions to Paleobiology No. 15: Permian Brachiopods of West Texas. II. By G. Arthur Cooper and Richard E. Grant. Pp. vii + 233-793 (plates 24-191). (Washington, DC: Smithsonian Institution Press, 1974. For sale by US Government Printing Office.) \$9.65. [295]
- International Labour Office. Geneva. Occupational Safety and Health Series, No. 26: Occupational Health Problems of Young Workers. By S. Forssman and G. H. Coppée. Pp. v + 143. (Geneva: International Labour Office, 1974.) [305]
- Australia: Department of Supply. Annual Report of the Defence Standards Laboratories 1972/1973. Pp. 88. (Maribyrnong, Victoria: Defence Standards Laboratories, 1974.) [305]
- Commonwealth Scientific and Industrial Research Organization. Australia. Annual Report 1972-73. Division of Animal Physiology. Pp. 85. (Melbourne: CSIRO, 1974.) [315]
- Endocrine Research Communications. Vol. 1 No. 1, 1974. Edited by Jerome Kowal. Pp. 1-116. Subscription: Vol. 1 (6 issues) \$35. (Students and Individual professionals \$15. (New York: Marcel Dekker Journals.) [315]
- Australian Journal of Plant Physiology. Vol. 1 No. 1, March 1974. Pp. xi + 1-175. Subscription \$A10 per annum. (4 issues) (Melbourne: Commonwealth Scientific and Industrial Research Organization, 1974.) [36]
- Government of India. Report of the Department of Agricultural Research and Education, 1973/1974. Pp. ii + 142. (New Delhi: Department of Agricultural Research and Education, Ministry of Agriculture, 1974.) [36]
- National Cancer Program. Report of the President's Cancer Panel, January 1973, submitted to the President of the United States. Pp. 15. (Bethesda, Md.: US Department of Health, Education and Welfare, Public Health Service, National Institute of Health, National Cancer Institute, 1974.) [36]
- Machine Diagnosis and Information Retrieval in Medicine in the USSR. (A publication of the Geographic Health Studies, John E. Fogarty International Center for Advanced Studies in the Health Sciences.) Pp. viii + 138. (Bethesda, Md.: US Department of Health, Education and Welfare, Public Health Service, National Institutes of Health, 1974.) [63]